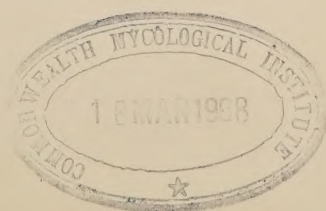




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PHILOSOPHICAL
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SERIES B

CONTAINING PAPERS OF A BIOLOGICAL CHARACTER.

VOL. 220.

*STUDIES IN THE BIOCHEMISTRY OF
MICRO-ORGANISMS.*

BY

PROFESSOR H. RAISTRICK IN COLLABORATION WITH

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THE Committee appointed by the *Royal Society* to direct the publication of the *Philosophical Transactions* take this opportunity to acquaint the public that it fully appears, as well from the Council-books and Journals of the Society as from repeated declarations which have been made in several former *Transactions*, that the printing of them was always, from time to time, the single act of the respective Secretaries, till the Forty-seventh Volume; the Society, as a Body, never interesting themselves any further in their publication than by occasionally recommending the revival of them to some of their Secretaries, when, from the particular circumstances of their affairs, the *Transactions* had happened for any length of time to be intermitted. And this seems principally to have been done with a view to satisfy the public that their usual meetings were then continued, for the improvement of knowledge and benefit of mankind: the great ends of their first institution by the Royal Charters, and which they have ever since steadily pursued.

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It is likewise necessary on this occasion to remark, that it is an established rule of the Society, to which they will always adhere, never to give their opinion, as a Body,

upon any subject, either of Nature or Art, that comes before them. And therefore the thanks, which are frequently proposed from the Chair, to be given to the authors of such papers as are read at their accustomed meetings, or to the persons through whose hands they received them, are to be considered in no other light than as a matter of civility, in return for the respect shown to the Society by those communications. The like also is to be said with regard to the several projects, inventions, and curiosities of various kinds, which are often exhibited to the Society; the authors whereof, or those who exhibit them, frequently take the liberty to report, and even to certify in the public newspapers, that they have met with the highest applause and approbation. And therefore it is hoped that no regard will hereafter be paid to such reports and public notices; which in some instances have been too lightly credited, to the dishonour of the Society.

PHILOSOPHICAL TRANSACTIONS.

Studies in the Biochemistry of Micro-organisms.

By HAROLD RAISTRICK and the Staff of the Biochemical Department, Nobel's Explosives Co., Ltd. (Imperial Chemical Industries, Ltd.), Ardeer Factory, Stevenston, Ayrshire, with Collaboration in Part IV by CHARLES THOM, United States Department of Agriculture, Bureau of Chemistry and Soils, Washington, and with Collaboration in Part XV by ROBERT ROBINSON, F.R.S., University College, London.

(Communicated by Sir FREDERICK HOPKINS, F.R.S.)

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PART I.—*Introductory.*

By HAROLD RAISTRICK and WILLIAM RINTOUL.

The application of fermentation processes to the arts, such as brewing, wine-making, the production of vinegar and the tanning process, has been practised from time immemorial, though only during the nineteenth century was it definitely recognized that the processes are accomplished by the aid of living organisms. To Pasteur chiefly is due the establishment of the necessity of the living cell to fermentation and the development of scientific practice to supersede the old rule-of-thumb methods.

In recent years an increasing interest is being taken in the biochemical investigation of micro-organisms and their products.

Of the micro-organisms which are known only the two classes—the Schizomycetes (bacteria) and the Eumycetes (true fungi)—seem to offer much hope of successful chemical investigation because of the relative ease with which they are cultivated in artificial media. Since more attention has hitherto been paid to the chemical activities of the Schizomycetes (bacteria) and of that family of Eumycetes known as the Saccharomycetaceæ (yeasts), it was decided to investigate first the metabolic products of other families of the Eumycetes popularly known as “moulds.”

Up to the present these fungi have been investigated chiefly from a morphological point of view. It is neither possible nor desirable here to deal with the mass of taxonomic literature running through two centuries of descriptive botany, but a review of the relatively few investigations dealing with the metabolic products of moulds will make it abundantly clear that certain families would justify chemical investigation with every hope of encountering new fermentation products or new types of fermentation.

Historical.

The formation of acid by moulds is so easily recognized by a simple titration of the medium that it is only natural that acid production early excited attention and most of the pioneer work on mould products deals with carboxylic acids which are formed in considerable amount by many of the commoner species of mould. The first acid to be encountered and recognized was oxalic acid, which is very easy to detect owing to the insolubility of its calcium salt.

Oxalic acid was detected in the tissues of moulds as crystals of calcium oxalate by many observers, but it was first recognized as a definite fermentation product by WEHMER (1891). In a series of classical researches he made an extended study of the conditions of its production from sugars by *Aspergillus niger*. By the addition of calcium carbonate to this medium, consisting of sugar and inorganic salts, he was able to obtain yields of calcium oxalate up to 120 per cent. of the sugar. Other observers have since confirmed and elaborated his findings, amongst whom are HEINZE (1903), EMMERLING (1903), CURRIE and THOM (1915) and ELFVING (1918).

Citric acid was first shown to be a product of mould fermentation by WEHMER (1893). He obtained it in excellent yield by growing species of *Citromyces* on synthetic media containing sugar as the only source of carbon. He again employed calcium carbonate to fix the acid and by this means claimed to obtain much improved yields. CURRIE (1917) investigated the lag between the total acidity and the oxalic acid produced by strains of black *Aspergillus* and this led him to the discovery that citric acid is produced in large amounts by this species. He showed that the formation of oxalic acid could be entirely inhibited by correct manipulation and by selecting the proper concentration of sugar in the medium, and worked out the conditions of citric acid manufacture from saccharose as a commercial process. Other observers, including MOLLIARD (1919) and BUTKEWITSCH (1922-4), have worked in the field of citric acid production. BLEYER (1926) has patented a process for its production in Germany, and FERNBACH, YUILL and ROWNTREE (1927) have developed a similar patent in this country.

Citric acid is now recognized as one of the commonest products of mould fermentation, as it is formed not only by species of *Citromyces* as was at first thought by WEHMER, but by many species of *Aspergillus*, and also by the majority of species of *Penicillium*, as is shown by CHRZASZCZ and TIUKOV (1929).

d-Gluconic acid was first isolated as a mould fermentation product by MOLLIARD (1922) from *Aspergillus niger*, and in a series of subsequent papers (1923, 1924) the conditions for the formation of gluconic, citric and oxalic acids were investigated, particularly with respect to unbalanced media. Partial deprivation of the inorganic constituents was found to lead to an increased yield of gluconic acid. MOLLIARD's discovery was soon confirmed by other workers, notably BERNHAUER (1924), BUTKEWITSCH (1924) and FALCK and KAPUR (1924). Still more recently MAY, HERRICK, THOM and CHURCH (1927) have shown that *d*-gluconic acid is formed in very high yield by *Penicillium luteum* var. *rubrisclerotium*, and in a later paper of HERRICK and MAY (1928) details are given of the preparation in quantity of gluconic acid from glucose by means of this organism.

Fumaric acid was first observed by EHRLICH (1911) to be a product of *Mucor stolonifer*, and was later shown by WEHMER (1918) to be formed in large amount from sugar by a species of *Aspergillus* which he named *A. fumaricus*. In a culture reputed to be *A. fumaricus* obtained from THOM we were unable to show any production of fumaric acid. It is of special interest, therefore, to note that WEHMER (1928) in a recent paper states that his culture, which originally gave large amounts of fumaric acid, now produces only traces of this substance and that gluconic acid is formed instead.

Malic acid.—Although casual references occur in the literature to the production of malic acid by moulds, there appear to be no well-founded proofs of its formation until the latest paper of WEHMER (1928). Here it is definitely shown that malic acid is produced in small amounts from glucose by *Aspergillus fumaricus*. We have been able to show that malic acid is produced by other moulds. It was isolated as the ester

from the acid products of one of the white species of *Aspergillus* (Ad. 55), (see Part IX), and also from *Aspergillus Wentii* (see Part XVII).

Succinic acid.—Here again there are isolated references to the occurrence of succinic acid as a mould product, without any definite evidence of its identification. We have found that it is actually formed in small amounts from sugar by several species of mould—*Aspergillus* and *Fumago* (see Parts IX and XVII). It was isolated as the ester and identified both as the free acid and by its derivatives. The formation of succinic acid by yeast in alcoholic fermentation has of course long been known, and it has been proved by EHRLICH (1909) to be derived from the de-amination of amino-acids. There is no evidence to show whether the acid formed by moulds is produced in a similar manner or is a direct product of the oxidative breakdown of sugar.

Other Mould Products.

Ethyl alcohol.—The formation of ethyl alcohol by moulds has been recognized in a few cases which have, however, until lately been regarded as exceptional. WEHMER (LAFAR : "Handbuch der technischen Mykologie") says, "Alcoholic fermentation is not found to any marked degree with any of the Aspergillaceæ, with one exception."

SANGUINETI (1897) observed alcohol production by *Aspergillus oryzae* from saccharose, starch and dextrin. ELFVING (1890) obtained yields of alcohol up to 4.2 per cent. from "*Penicillium glaucum*." MAZÉ (1902) regarded alcohol as a normal intermediate product in the breakdown of sugar by moulds and instanced its formation by *Allescheria gayoni*. We have shown that large groups of *Aspergilli* and *Penicillia* are capable of producing alcohol in quantity and have used this characteristic as one means of division into sub-groups for classification purposes (see Parts III and IV). A recent paper by YUILL (1928) points out that alcohol is formed by *Aspergillus flavus*. This mould belongs actually to the group of *Aspergilli* producing the highest yields of alcohol. Species of *Fusarium* were also recognized as being pre-eminently alcohol-formers; ANDERSON and WILLAMAN (1922) showed this to be the case for *Fusarium lini*, and WHITE and WILLAMAN (1928) compared the mechanism of fermentation by *Fusarium lini* with that of yeast. The production of alcohol by *Fusaria* is abundantly confirmed in our studies on this group (see Part V).

Acetaldehyde.—NEUBERG and COHEN (1921) found that acetaldehyde was produced from sugar by various moulds, including several species of *Aspergillus* and one species of *Penicillium*. The acetaldehyde was "intercepted" by the addition of a sulphite, as in the production of glycerol by yeast fermentation, and was in some cases obtained in fair yield. The addition of sulphite, however, causes a profound modification of the fermentation process.

We have found in several cases of moulds producing alcohol that, without any interceptor present, acetaldehyde can be isolated as the 2:4-dinitrophenylhydrazone from the volatile products (see Part XVII).

Mannitol.—Although the presence of mannitol in the mycelium of fungi has long

been recognized, it has hitherto never been regarded as a definite fermentation product of moulds. We have been able to show that it is produced by many moulds, and by certain of the white species of *Aspergillus* in particular, in yields as high as 50 per cent. of the sugar fermented. An account of the production of mannitol is given in Part IX and it need not, therefore, be considered here.

Polysaccharides.—The recorded polysaccharides from the lower fungi are comparatively few. CRAMER (1894) obtained from "*Penicillium glaucum*" spores a carbohydrate hydrolysing completely to glucose and giving a blue colour with iodine. He named it "spore starch." ALSBERG and BLACK (1913) obtained a similar substance from *Penicillium puberulum*. It gave a violet colour with iodine and was considered to be identical with trehalum from manna. DOX and NEIDIG (1914) isolated from *Penicillium expansum* a substance, mycodextran, giving glucose on hydrolysis and no colour with iodine. Later (1914 (2)) they isolated from *Aspergillus niger* another carbohydrate, mycogalactan, which gave galactose on hydrolysis.

BOAS (1917, 1919, 1922) examined in some detail the polysaccharide obtained from *Aspergillus niger* when grown in acid media at a fairly high temperature. It gives a blue colour with iodine and is dextro-rotatory, $[\alpha]_D = +120^\circ$ – 160° . It is formed from various sugars, polyhydric alcohols and carboxylic acids. LAPPALAINEN (1919) examined more closely the conditions of its formation, and further work on this same "mould starch" is described by SCHMIDT (1925). She finds that it is identical with amylose and therefore with isolichenin.

In the present series of studies on moulds, several polysaccharides have been encountered and they seem to be formed in some cases in considerable amount. A substance resembling, if not identical with, glycogen was isolated from one of the white *Aspergilli*, Ac. 56 (see Part IX). *Fumago vagans* has been shown to synthesize in fair quantity a polysaccharide, hydrolysing to dextrose and giving no colour with iodine (see Part XVII), while *Penicillium digitatum* produces a different polysaccharide having a very high dextro-rotation, and giving no colour with iodine (see Part XVIII). The most interesting product, however, is that formed by *Penicillium luteum* and described in detail in Part XIII. This product, which we have called "Luteic acid," is a complex, built up of units each consisting of two molecules of glucose to one of malonic acid in such a manner that one of the carboxyl groups is still free. It may be described as a malonyl-polyglucose. It is lævo-rotatory and gives rise to viscous solutions, this viscosity of the medium being characteristic of the mould in question. Yields of 12 per cent. of this material can be readily obtained. The product most resembles some of the soluble specific substances isolated by HEIDELBERGER and GOEBEL (1927) from *Pneumococcus*.

The reason for the elaboration of these complex substances by the mould organism has not been elucidated, the most likely hypothesis being that they are merely reserve foodstuffs stored in a less soluble, non-dialysable form and therefore more easily retained for eventual requirements than the simpler sugars.

Fats.—Although fats derived from yeast have been isolated and investigated, very little work seems to have been devoted to the fats formed by moulds. The earliest observers, NAEGELI and LOEW (1878), and SIEBER (1881), seem to have worked with cultures of doubtful authenticity and purity. There are some recent observations which are of greater interest. In 1921 a German patent was taken out relating to fat production from carbohydrates. The fat in this case is obtained by inoculating turnips, apples, &c., with certain specified fungi. BELIN (1926) refers to the production of fat by *Aspergillus niger*, and BARBER (1927) describes the isolation of fat from a species of *Penicillium* grown on sucrose solution. TERROINE (1927) is concerned with the formation of fat by *Aspergillus niger* from sugar chiefly from an energy standpoint. The function of the fat formed is obscure. It may be merely a reserve foodstuff which is accumulated in the same way as the polysaccharides. Our own observations have not included the fats, although a most interesting and new complex fatty acid, produced by *Penicillium spiculisporum*, has been isolated and investigated. It is described in Part XVI of this series.

Phenolic Bodies.

(a) *Kojic acid*.—This curious substance—5-hydroxy-2-hydroxymethyl- γ -pyrone—was first isolated by YABUTA (1912), although its colour reaction with ferric chloride had previously been noted by SAITO (1907). It is formed by various species of the *Aspergillus flavus-oryzae* group and its production has indeed been used by us as diagnostic of this group. Several observers since YABUTA have described new mould products which from their properties would appear to be identical with kojic acid. Kojic acid forms the subject of two papers in this series—Parts VII and VIII.

(b) *Penicillic acid*, $C_8H_{10}O_4 \cdot 2H_2O$, and *mycophenolic acid*, $C_{17}H_{20}O_6$, were two products of phenolic type obtained by ALSBERG and BLACK (1913) from *Penicillium puberulum* and *Penicillium stoloniferum* respectively. Both these moulds were obtained from spoiled maize. Apart from determining the empirical formulæ and noting their reactions and toxicity towards certain animals, nothing further appears to have been done with these products, their constitution still remaining to be elucidated.

(c) Our own observations have led to the discovery of several new phenolic bodies. A yellow benzopyrone derivative to which we have given the name "Citromycetin," and which has the formula $C_{14}H_{10}O_7 \cdot 2H_2O$, is produced only by certain species of *Citromyces*. The yields of this material are really good and may under certain conditions reach 25–30 per cent. of the glucose fermented. The preparation and constitution of citromycetin are dealt with in Part XI of this series. Another yellow colouring matter, having the empirical formula $C_{13}H_{14}O_5$ to which we have given the name "Citrinin," is a metabolic product of *Penicillium citrinum*. Yields approximating 5 per cent. of the sugar fermented were obtained. This material is discussed in Parts XIV and XV. A purple-coloured quinone having the empirical formula $C_8H_4O_5$ and the chemical

constitution of a methoxy-dihydroxy-toluquinone is produced by certain strains in the *P. spinulosum* series. This material is described in Part XII of this series.

When it was decided in 1922 to commence a comprehensive scheme of work on the general biochemistry of micro-organisms, and the "moulds" were chosen as the first group of micro-organisms for investigation, it at once became evident that with such a wealth of fungi available it would be impossible to investigate in detail, in any reasonable space of time, the chemical compounds formed by even a small proportion of the different known species of fungi. For this reason then, instead of attempting the isolation and identification of the compounds formed by any mould taken at random, it was decided to investigate quantitatively the *types* of compounds formed by each of a large number of fungi, so as to obtain a logical basis for the choice of any particular fungus for later investigation. To this end a method involving the preparation of carbon balance sheets was evolved and is described in detail in Part II of this series. This method is capable of being applied to any organism which will grow in or on synthetic media, and hence is applicable to bacteria as well as to moulds. It gives a clean-cut classification of the various types of products formed and of their quantitative relationships. In order further to reduce the scope of the work and to standardize conditions so as to ensure the possibility of repeating the work, all experiments were carried out using glucose as the sole source of carbon and a CZAPEK-DOX synthetic medium having the following composition was used as the basal metabolism solution:—

| | | | | | | | | |
|--------------------------------------|----|----|----|----|----|----|-------|------|
| Glucose | .. | .. | .. | .. | .. | .. | 50 | gm. |
| NaNO ₃ | .. | .. | .. | .. | .. | .. | 2 | " |
| KH ₂ PO ₄ | .. | .. | .. | .. | .. | .. | 1 | " |
| KCl | .. | .. | .. | .. | .. | .. | 0.5 | " |
| MgSO ₄ .7H ₂ O | .. | .. | .. | .. | .. | .. | 0.5 | " |
| FeSO ₄ .7H ₂ O | .. | .. | .. | .. | .. | .. | 0.01 | " |
| Water | .. | .. | .. | .. | .. | .. | 1,000 | c.c. |

By means of the carbon balance sheets it has been possible to eliminate from further investigation all those fungi—constituting by far the greater number—which, under the conditions of our experiments, produce practically nothing else but carbon dioxide from glucose, and thus very greatly to economize time, by only attempting the isolation and identification of end products from a comparatively small number of fungi already proved from their carbon balance sheets to be biochemically interesting, and to give rise to considerable yields of some end product other than carbon dioxide. The quantitative results and carbon balance sheets obtained with 96 species of *Aspergillus* are given in Part III, from 75 species of *Penicillium* and 8 species of *Citromyces* in Part IV, from 23 species of *Fusarium* in Part V, and from 36 miscellaneous species of fungi in Part VI.

Having, by means of the carbon balance sheets, made a choice of species suitable for further intensive investigation, these chosen species were now grown on a larger scale

and their metabolic products investigated. For this purpose an apparatus was designed for the cultivation of fungi in pure culture on a large laboratory scale. This apparatus is described in Part VII.

Cultures.

It was realized at the commencement that in work of this description no pains should be spared to ensure the authenticity and purity of all cultures used. To this end the strictest care was exercised, not only in the choice of cultures for examination but also in sub-culturing them subsequently in the laboratory, in order to avoid errors in nomenclature. Immediately on receipt, all fungi were examined and single-spore cultures prepared from them, so that all the work described was carried out on single-spore cultures.

Most of the cultures used have been obtained from one of the following sources :—

1. Microbiological Laboratory of the United States Department of Agriculture.
2. The American Type Culture Collection at Chicago.
3. Centraalbureau voor Schimmelcultures at Baarn, Holland.
4. PRIBRAM'S Mikrobiologische Sammlung in Vienna.
5. The National Collection of Type Cultures, Lister Institute, London.

The remainder of our cultures were either isolated by ourselves at Ardeer or were received from private donors.

Considerable difficulty was experienced from time to time with cultures purchased from certain of the above sources of supply, since some of the cultures were obviously incorrectly named. In these circumstances it would have been impossible to carry through the work on the *Aspergilli* and *Penicillia* without the whole-hearted co-operation of Dr. CHARLES THOM of the Microbiological Department of the United States Department of Agriculture. We desire to offer to him and to his colleague, Miss MARGARET B. CHURCH, our warmest thanks for their kindly co-operation.

We are also greatly indebted, for expert advice on the cultures of *Citromyces* on which we have worked, to Prof. CARL WEHMER of the Technical High School of Hanover, and to Prof. PHILIP BOURGE of the University of Louvain, to both of whom we offer our best thanks.

Practically all the species of *Fusarium* used were WOLLENWEBER'S original cultures obtained from the Centraalbureau at Baarn, and hence should be above suspicion.

We have received several different species of fungi from Mr. F. T. BROOKS, F.R.S., of Cambridge, for which, and for the keen interest he has shown in this work since its inception, we desire to thank him.

Sub-cultures of the whole of the cultures worked upon and described in this series of papers have been deposited with the National Collection of Type Cultures at the Lister Institute, London. We wish to express our indebtedness to the Medical Research Council for their kindness in making this possible.

The main results arising from these observations may be briefly summarized as follows :—

1. The carbon balance sheets may be used as a biochemical method for the classification of different species in certain families of fungi. This is particularly true in the case of species of *Aspergillus*. In this genus the different species arranged according to their biochemical characteristics follow closely the classification based on morphological characteristics and used by Dr. THOM (1926) in his recent book 'The *Aspergilli*.' With species of *Fusarium*, on the other hand, there seems little hope of classification on these lines, since all the species tested gave rise principally to alcohol and showed themselves closely allied to the *Saccharomyces*.

2. The experimental data seem to throw some light on the biochemistry of the initial stages of the breakdown of the glucose molecule by fungi. It appears that the first stage is a CANNIZZARO reaction involving the production from two molecules of glucose of one molecule of mannitol and one molecule of gluconic acid. Depending then on whether the mould in question prefers for growth an acid or a neutral medium, either mannitol or gluconic acid (of course, in some cases both) is destroyed. There is ample evidence to support this view. The white *Aspergillus*, Ac. 55, which gives rise to yields of mannitol approximating to but never exceeding 50 per cent. of the glucose metabolized (see Part IX), produces very little if any gluconic acid, refuses to grow on an acid medium, and even when cultivated on a medium with an initial pH of 4·6, changes this during the course of its growth to 6·5 to 7·0. On the other hand, species which produce large quantities of gluconic acid, e.g., *Aspergillus Wentii* and *Penicillium chrysogenum* (Catalogue No. Ad. 11), give small amounts of mannitol, on which material they grow quite well, but bring the pH of the medium to about 1 to 2 no matter what the original pH may be. Other species, again, produce moderate amounts of both mannitol and gluconic acid. The production of these two materials from glucose raises an interesting stereochemical point. Glucose on reduction should give sorbitol, and on oxidation gluconic acid, while, on the other hand, mannitol arises from mannose, which gives mannonic acid on oxidation. The curious fact remains, however, that in spite of frequent search for sorbitol and mannonic acid as metabolic products of fungi, not even a trace of them has been found, although yields of 50 per cent. of mannitol or gluconic acid are easily obtained.

3. Perhaps the most striking fact arising from these investigations is the extraordinary specificity of the different mould products. This has led us to the belief that while such general biochemical reactions as the production from glucose of mannitol and gluconic acid, of ethyl alcohol, of citric and oxalic acids may be regarded as common to many species belonging to many different genera of fungi, there are certain highly specific products which are only produced, in some cases by a single species, and in others by a very small sub-group containing a very few species. Thus, for example, the new product, citrinin, described in Part XIV, is produced only by *Penicillium citrinum* THOM. This is so striking that the cultures of *Penicillium citrinum* in our

collection may be distinguished from all the other five or six hundred species belonging to many different genera, by the purely chemical test of adding ferric chloride to their metabolism solutions, when the colour reaction characteristic of citrinin enables one to pick out cultures of *P. citrinum* from any others. Similarly citromycetin, which is described in Part XI, is produced only by species of *Citromyces*, while the methoxy-dihydroxy-toluquinone, described in Part XII, is produced only by certain strains in the *P. spinulosum* series. Other examples will be found in Parts VII, XIII, XVI and XVIII.

4. The amazing powers of the fungi of bringing about *synthetic* chemical processes is shown from a consideration of the composition and constitution of some of the above specific products. While the general mould products such as citric, fumaric and oxalic acids, ethyl alcohol, &c., are simple breakdown products, the specific products are almost invariably much more complex than the original glucose molecule. The methoxy-dihydroxy-toluquinone ($C_8H_8O_5$) already mentioned is among the simplest of these products. On the other hand, citromycetin ($C_{14}H_{10}O_7 \cdot 2H_2O$) contains a benzo-pyrone nucleus, while citrinin ($C_{13}H_{14}O_5$), the exact constitution of which is given in Part XV, contains a benzene ring to which is fused another ring containing oxygen. The product ($C_{17}H_{28}O_5$) given by *Penicillium spiculisporum* and described in Part XVI, is of an entirely different type. It is the lactone of a monohydroxy-tricarboxylic fatty acid having a straight chain of 15 carbon atoms. Finally, the colloidal material luteic acid produced by *Penicillium luteum* is a malonyl-polyglucose, somewhat similar in general chemical structure to acetyl cellulose.

5. There is a general resemblance between some of the specific products given by the lower fungi and various members of the so-called lichen acids. Thus the product from *P. spiculisporum* ($C_{17}H_{28}O_5$) is similar both in empirical formula and general chemical properties to oxyroccellic acid ($C_{17}H_{32}O_5$), isolated by HESSE (1898) from different species of the lichen *Roccella* and to proto- α -lichesterinic acid ($C_{18}H_{30}O_5$) which occurs in the lichen *Cetraria islandica*. This resemblance seems to support the view that it is the fungal part of the fungus-alga symbionts constituting the lichen which is responsible for the formation of the lichen acids.

This work was originally undertaken after discussion with Sir FREDERICK GOWLAND HOPKINS, and we would offer to him our thanks for his continued interest in it during the seven years it has been in progress. We desire also to express our appreciation of the attitude adopted by the Directorate of Messrs. Nobel Industries Limited, in originally sanctioning the undertaking, and our thanks to the Directorate of Imperial Chemical Industries Limited for permission to publish the work. Finally, our best thanks are due to our assistants, Messrs. J. J. TIDD, R. H. BARNETT, H. B. COLQUHOUN, A. CURRIE, J. PATON and R. PAGE, without whose loyal help it would have been difficult to carry out what was at times very laborious work.

Studies in the Biochemistry of Micro-organisms.

PART II.—*Quantitative Methods and Technique of Investigation of the Products of Metabolism of Micro-organisms.*

By JOHN HOWARD BIRKINSHAW and HAROLD RAISTRICK.

At the commencement of this work it was decided, as outlined on p. 7 in the introductory paper in this series, to take as the basis of the investigations the quantitative determination of the *types* of compounds formed by micro-organisms. Hence it became necessary to devise methods of separating the products of metabolism arising from glucose into different *types* of chemical compounds, and of isolating them in a form suitable for the subsequent estimation of their carbon content by combustion. The methods adopted will be described.

(A) *The estimation of carbon by wet combustion.*

Several methods have been described from time to time for the estimation of carbon in solution, but the principle involved appears to be the same in almost all, namely, oxidation of the carbon compound with chromic acid, and estimation of the carbon dioxide produced. In the method reported by GREY (1914) the carbon dioxide is estimated by measuring its volume. In other methods it is customary to follow the classical method of estimating carbon by dry combustion, and weighing the carbon dioxide formed. The method finally adopted in this work is a modification of that of MESSINGER (1890). It consists in the oxidation of the carbon compounds by a mixture of sulphuric and chromic acids. The chief product of oxidation is carbon dioxide, but the combustion gases are passed over heated copper oxide to complete the oxidation of any carbon monoxide produced, and also of any volatile carbon compounds which have escaped oxidation by the combustion mixture. The carbon dioxide is absorbed in standard baryta solution and estimated by titration.

The apparatus used is shown in fig. 1 and consists of three parts.

(1) *Purification of air current.*—The air is purified by the usual arrangement of aspirators (A) for holding air, and of soda-lime towers (B1 and B2) for removing carbon dioxide.

(2) *Combustion.*—The substance is oxidized in the 250 c.c. flask F, which is connected by the ground-glass joint and three-way tap T₁ to the combustion tube CC₁, and to the air by the three-way tap V, thus providing alternative air paths via the funnel H or the tube G. The combustion tube CC₁ is filled for about half its length with granular copper oxide, and for about one-third with lead chromate, a space being left at the end C₁ for the insertion of a copper spiral, which is to be employed if nitrates are present in the solution to be oxidized.

(3) *Absorption of carbon dioxide.*—The carbon dioxide is absorbed in the train of baryta bubblers N, O, P, which contain suitable measured amounts of N/4 baryta. P is of 25 c.c., O of 50 c.c., and N of 50 or 100 c.c. capacity, depending on the amount of carbon dioxide anticipated. The bubblers are connected to the combustion apparatus by the three-way tap T_2 . There is thus an alternative air path through the indicator baryta bubbler M.

The combustion is carried out as follows :—10 c.c. of chromic acid solution (100 gm. of chromic anhydride in 100 c.c. solution) are pipetted into flask F, which is then replaced. The combustion tube CC' is heated and CO_2 -free air passed through the whole apparatus

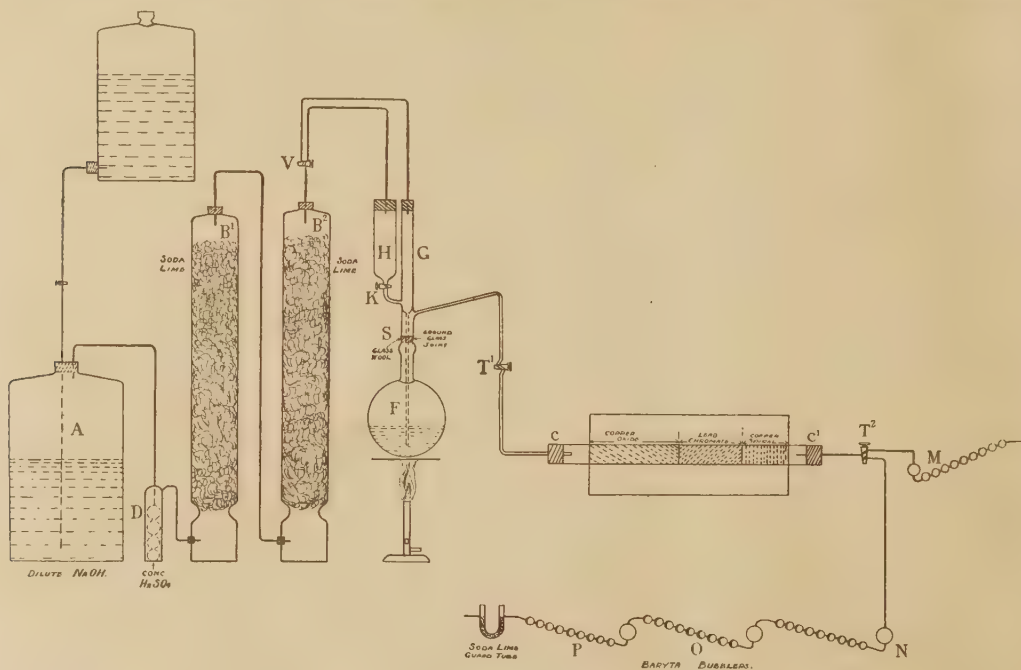


FIG. 1

from A, via tube G, until free from carbon dioxide, as tested by bubbling the air for a short time through M. V is then closed and T_2 connected with the bubblers N, O, P. K is closed, the stopper from H removed, and a suitable amount of the solution to be oxidized is pipetted into H. It is run into F by replacing the stopper in H, connecting V to H and opening K. In a similar way the solution is washed into F, first with a little water, and then with 100 c.c. of concentrated sulphuric acid, divided into three portions and added, by means of a pipette, to wash down the sides of H. A slow stream of air is passed via G through F, which is heated, gently at first and later more strongly, to bring about combustion. When this is complete, heating is stopped and the combustion gases are swept out of the apparatus and through the bubblers N, O, P by a quicker current of air.

If a solid is to be oxidized the procedure is similar, except that the substance is weighed out directly into F at the commencement, and the chromic acid is only run from H after the apparatus has been initially filled with CO₂-free air, and before the sulphuric acid is introduced.

At the end of the combustion the bubblers containing any barium carbonate—usually only N, but occasionally O—are washed out and titrated with N/2 hydrochloric acid to phenolphthalein. The baryta in P should always remain perfectly clear, but if any cloudiness appears another bubbler is immediately added in order to ensure complete absorption.

Blank estimations are, of course, necessary, as both chromic acid and sulphuric acid nearly always contain a little carbon.

To test out the method, a number of carbon estimations were made on a variety of pure substances. The results, which are given in Table 1, indicate that the method is capable of giving perfectly accurate and reliable results. It is also decidedly economical in time.

TABLE 1.—*Estimation of carbon in pure substances by wet combustion.*

| Substance analysed. | Percentage Carbon. | |
|---|-------------------------|---|
| | Found. | Calculated. |
| Cane sugar | 42·43 41·75 | 42·11 |
| Acetaldehyde ammonium bisulphite | 19·11 | 19·18 |
| Oxalic acid | 18·85 | 19·04 |
| Fumaric acid | 41·11 | 41·37 |
| Succinic acid | 40·91 | 40·68 |
| Mucic acid | 34·36 | 34·27 |
| Acetone, 5 c.c. of stock solution containing 0·2437 gm. acetone . . | 62·21 | 62·00 |
| Silver acetate | 14·47 14·33 14·45 | 14·38 |
| Acetic acid (10 c.c. of solution) | 0·1121 gm. | 0·1120 gm. calculated from titration. |
| Dry mycelium of <i>Aspergillus niger</i> | 46·86 46·71 | 46·55 by direct dry combustion. |

(B) *Apparatus for metabolism experiments.*

It is evident that in work of this nature, involving the culture of micro-organisms with a view to the subsequent analysis of their metabolic products and the preparation of carbon balance sheets for each organism studied, a suitable type of apparatus must fulfil the following conditions :—

- (1) It must permit of the organism being grown in a closed system so that the whole of the metabolic products—gaseous, liquid and solid—are retained in such a manner as to be easily subjected to quantitative examination.
- (2) It must allow of the passage of a continuous stream of sterile gas (air, nitrogen, &c.) over the surface of, or through, the liquid where the organism is growing, so that the experiment may be conducted aerobically or anaerobically at will, and so as to enable the gaseous products of metabolism to be collected for analysis.

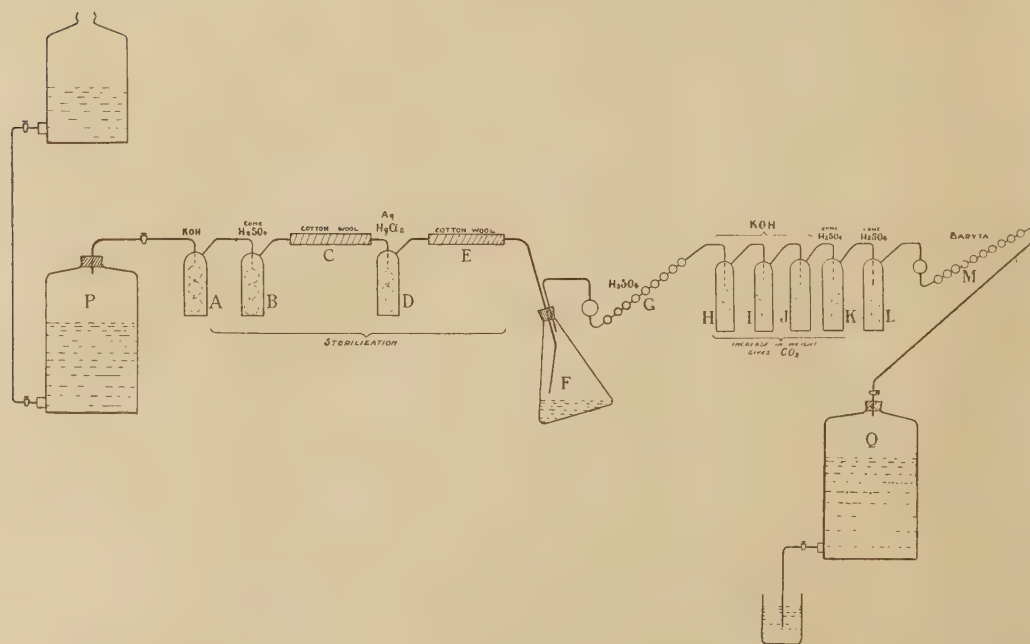


FIG. 2

The form of apparatus finally decided on is shown in fig. 2. It was designed primarily for the cultivation of fungi but, with very slight modification, it is suitable for use with any type of micro-organism.

It consists of three parts designed for :—

- (1) *Purification of the gas current.*—Whatever gas is used must be microbiologically sterile and chemically free from carbon dioxide. This is done by passing the gas first

through a potassium hydroxide bubbler A to remove carbon dioxide, then through a concentrated sulphuric acid bubbler B and a sterilized cotton-wool filter C. The gas is further sterilized by bubbling through dilute mercuric chloride solution (1 : 1,000) in D and finally through another sterilized cotton-wool filter E. The bubbler D serves a double purpose. Since it contains mercuric chloride solution, it helps to sterilize the gas in addition to maintaining saturation of the gas with moisture, thus avoiding evaporation of the contents of flask F. The efficiency of the sterilization effected by the above method is shown by the fact that, although relatively large volumes of air are passed through the culture flask F during the course of an experiment, often lasting several weeks, cases of infection have been extremely rare.

(2) *Cultivation of the micro-organism.*—The culture being studied is grown on a suitable culture medium in a 750 c.c. conical flask F, the neck of which is closed by a sterile bung, carrying an inlet glass tube which reaches practically to the surface of the culture medium, and an outlet tube, the end of which only just passes through the bung. Each end of each tube is fitted with a cotton-wool plug, and the whole fitting—bung and glass tubes—is sterilized in the autoclave immediately before use.

(3) *Absorption and collection of the gaseous end-products.*—The gas stream, as it emerges from the flask F, contains carbon in the form of carbon dioxide and traces of volatile organic compounds. The latter are removed by passing the gas through the sulphuric acid bubbler G, which also serves to dry the gas before absorption of the carbon dioxide in the absorption bulbs—three potassium hydroxide bulbs H, I, J and a concentrated sulphuric acid bulb K. These four bulbs are weighed at the beginning and at the end of an experiment, the difference giving the weight of carbon dioxide formed. The bulb L contains concentrated sulphuric acid to protect the bulb K from back diffusion of water vapour. The baryta bubbler M is merely an indicator to show whether absorption of carbon dioxide is complete. A known volume of standard baryta is measured into M at the start of each experiment, so that if any carbon dioxide has escaped absorption in the potassium hydroxide bulbs, as evidenced by the appearance of a turbidity in M, it may be readily estimated by titration. The gaseous products of metabolism, other than carbon dioxide, together with the residual gases passed through F, are collected in the aspirator Q.

(C) *Technique employed for the cultivation of micro-organisms in the metabolism experiments.*

In the metabolism experiments described in Parts III, IV, V and VI it was essential that the medium employed for the cultivation of the fungi should be a purely synthetic one, so as to admit of reproduction at any future time. The medium finally chosen for use was that recommended by CZAPEK (1902) and later by DOX (1910) and has the composition given in Part I, page 7. It is generally suitable as a standard medium for the cultivation of fungi and is largely used for that purpose. It has only one source

of carbon, has a standard chemical composition, is easily reproducible, and lends itself admirably to analysis.

The medium was made up in large batches of 10–15 litres, and was then accurately pipetted out, in 250 c.c. quantities, into a large number of 750 c.c. conical flasks, all of the same shape. The flasks were then plugged with cotton-wool, and sterilized by steaming for half an hour on each of three consecutive days. Three flasks from each batch were taken for analysis, and the remainder were covered with lead-foil caps to prevent evaporation, and stored for use. Under these conditions the medium kept perfectly, as, even at the end of twelve months' storage at 24° C., analysis failed to reveal any alteration in the carbohydrate content. The flasks were always stored at 24° C. for at least a month before use, in order to detect any which had been imperfectly sterilized.

A number of similar-sized fittings for the necks of the culture flasks—comprising rubber bung and glass delivery tubes, as described on p. 15—were made, so that each would fit any of the culture flasks. These fittings were sterilized before use by wrapping in parchment paper and heating in an autoclave at 120° C. for an hour. Fresh cotton-wool plugs were inserted in the side tubes for each experiment.

At the commencement of a metabolism experiment one of the 750 c.c. culture flasks is sown with spores of the organism under investigation. This is done by emulsifying, in 5 c.c. of sterile distilled water, the spores of a young culture grown at 24° C. on a test-tube slope of CZAPEK-Dox agar, and adding the emulsion to the medium. This mass sowing, which experiment showed to add only negligible traces of carbon to the medium in the metabolism flask, was chosen in preference to the more usual small sowing, in order to encourage quick growth of a mycelial felt and minimize the risk of infection. In practice it worked very well, especially with the more "delicate" fungi, which, with a small sowing, give only a very feeble growth and are often contaminated. Single-spore cultures of all the fungi investigated were used in every experiment described.

Immediately after sowing, the sterilized rubber bung and side tubes are inserted in the neck of the culture flask in place of the cotton-wool plug, and the flask is inserted in position in the apparatus for these metabolism experiments described on p. 14. A bubbler G, containing 20 c.c. of fresh concentrated sulphuric acid, the carbon dioxide absorption bulbs freshly charged and weighed, and an aspirator Q filled with water are also inserted in their proper places. The whole apparatus is tested for gas leaks and is then incubated for a suitable period, varying from two to eight weeks.

The whole of the metabolism experiments were carried out in a large constant-temperature room maintained at an average temperature of 24° C. (23°–25°), as many as 14 experiments proceeding concurrently.

During the whole of the experiments described in Parts III, IV, V and VI air was the only gas passed through the metabolism apparatus. At first, aspirators were used to supply the air, but later, with the expansion of the work, it became desirable to

use compressed air. This was drawn from taps laid at convenient intervals in a compressed-air supply pipe which circled the incubator room. The method of aeration used at first was to bubble a slow stream of air continuously through the whole apparatus. Later, however, this was abandoned, as it was found that more interesting results were obtained by passing about 400–500 c.c. of sterile air through the apparatus during the space of one hour per day. This method of “controlled aeration” is probably responsible for a number of the interesting results obtained—especially with the *Aspergilli* and *Penicillia*—a fact which is discussed in greater detail in Parts III and IV.

At the end of a metabolism experiment, which is stopped, if possible, before all the glucose has disappeared, in order to minimize destruction of possible metabolism products, a quick current of air is passed through the whole metabolism apparatus for two or three hours. The culture flask F is taken off for examination and analysis, and the various parts—the sulphuric acid bubbler G, the carbon dioxide absorption bulbs H, I, J, K, and the aspirator Q—are disconnected and their contents submitted to analysis. In all cases portions of the mycelium are immediately transferred to CZAPEK-DOX agar and beer-wort agar and incubated at 24° C. to test the purity of the culture, while another portion is examined microscopically for contaminations.

(D) *Methods of separation and analysis of the metabolic products.*

The carbon originally supplied to the fungus as glucose has been metabolized by it, at the end of a metabolism experiment, into a number of different types of carbon compounds, which, for ease of subsequent description, may be divided first into three different classes :—

- (1) Carbon as gaseous compounds.
- (2) Carbon as insoluble solid compounds.
- (3) Carbon as compounds in solution.

These will be dealt with in turn, and the methods of separation and analysis for each class described as they arise.

(1) *Carbon as gaseous compounds.*—All the gaseous products of metabolism are contained in the sulphuric acid bubbler G, in the potash bulbs H, I, J, in K, and in the aspirator Q. The carbon as gaseous products may thus be subdivided into three sub-classes.

(1a) *Carbon as volatile compounds soluble in strong sulphuric acid.*—This sub-class includes small amounts of the vapours of such bodies as the alcohols, aldehydes, ketones, &c. These are carried over by the stream of air passed during the experiment, and are absorbed in the strong sulphuric acid contained in bubbler G. Its carbon content is estimated by wet combustion in the combustion apparatus.

(1b) *Carbon as carbon dioxide.*—The four bulbs H, I, J and K are disconnected, wiped clean, allowed to stand in the balance room for an hour and weighed. From the weight of carbon dioxide the carbon as CO₂ is readily calculated.

(1c) *Carbon as permanent gases*.—The gas in the aspirator Q, consisting of vitiated air together with any gaseous products, other than carbon dioxide, arising from the decomposition of the sugar by the fungus, is brought to atmospheric pressure in the incubator room by running water into the aspirator until the pressure is adjusted. The temperature and atmospheric pressure are then noted. The volume of the gas is determined, after analysis, by deducting the volume of the residual water from the volume of the aspirator. The gas is then analysed in a BONE-WHEELER gas-analysis apparatus for—

(1) *Oxygen*—which is absorbed in alkaline pyrogallol in the usual way.

(2) *Combustible gases*—e.g., methane, hydrogen.

These gases are first tested for qualitatively by adding to a known volume of the vitiated air from the aspirator a known volume of explosive gas—made by the electrolysis of acidulated water, and consisting of a mixture of hydrogen and oxygen in the proportion of two volumes of hydrogen to one volume of oxygen. The function of this explosive gas is to initiate the explosion of any methane or hydrogen which may be present. The mixture of vitiated air and explosive gas is then exploded in the BONE-WHEELER apparatus and the volume of residual gas noted. Unless this volume is less than the original volume of vitiated air taken for analysis no methane, hydrogen or other combustible gas can be present. This follows, since an electrolytic explosive gas leaves no residue on explosion, and since, in addition, methane, hydrogen or any other combustible gas likely to arise during fermentation shrinks in volume on exploding with oxygen, as is evident from a consideration of the various equations.

No evidence has so far been obtained of the production of either methane or hydrogen, or of any other combustible gas, by any species of mould tested.

The main use at present of the analysis of the vitiated air is that it makes it possible to estimate the “respiration coefficient” for the fungus under investigation. The respiration coefficient, which is $\frac{\text{Volume of carbon dioxide produced}}{\text{Volume of oxygen absorbed}}$, has proved of considerable value for classification purposes, particularly with the *Aspergilli* and *Penicillia*.

The volume of carbon dioxide produced is readily calculated from the sum of the carbon dioxide absorbed in the potash bulbs (1b) and the carbon dioxide dissolved in solution (3b).

The volume of oxygen absorbed is also readily calculated by making what appears to be a justifiable assumption, at any rate with any of the fungi used. It is assumed that these fungi do not fix atmospheric nitrogen nor produce nitrogen from the sodium nitrate contained in the medium. Since the final volume of vitiated air and its content of oxygen are known, the final volume of nitrogen can be calculated. Making the above assumption, this volume is also equal to the volume of the nitrogen present in the air originally passed through the apparatus. The volume of this air can be readily

calculated from the volume of nitrogen by using the factor 79.03 per cent. for the percentage of nitrogen in atmospheric air. Then, by deducting from the original volume of air the volume of vitiated air at the finish, the volume of oxygen absorbed is obtained. This figure is probably slightly inaccurate, but not sufficiently so to affect the respiration coefficient appreciably. Since very large differences are observed in the respiration coefficients in different species of fungi this slight error becomes quite negligible in comparing the respective respiration coefficients.

(2) *Carbon as insoluble carbon compounds.*—The only substance included in this class which has so far been encountered is the mycelium of the fungus under observation. A little difficulty was experienced at first in separating satisfactorily the mycelium from the metabolic products in solution. This was due to the fact that it was found in earlier experiments that, when a mould mycelium is filtered through an ordinary filter paper, it is impossible, after drying, to detach the mycelium and the accompanying spores in any quantitative fashion without detaching fibres of filter paper, which vitiate the carbon figures obtained in the subsequent combustion of the mycelium. In order to overcome this difficulty recourse was had to filtration through kieselguhr, since the fact was established by experiment that, on drying a filter paper which has been covered during filtration with a thin layer of kieselguhr, the latter peels off in a layer without detaching any fibres from the paper.

The procedure adopted to separate the mycelium from the soluble metabolic products is as follows :—

The metabolism flask F is detached from the apparatus and, after mycological examination (see p. 17), the contents are neutralised to pH 7.0 with standard sodium hydroxide (or hydrochloric acid), using bromthymol blue paper as an external indicator. This titration gives the acid (or alkali) formed by the organism, since the titration value, to the same pH, of the original medium is known. The liquid is then filtered through kieselguhr in the following way.

An 11 cm. Buchner funnel with a double, well-fitting filter paper is connected to an Irvine filtering tube (1915). About 0.2 gm. of kieselguhr, which has previously been thoroughly extracted with concentrated hydrochloric acid, then washed, calcined and preserved dry, is accurately weighed into a 500 c.c. flask, and a smooth suspension made by shaking with 300–400 c.c. of distilled water. This is poured on the filter paper so as to give a uniform layer, the flask being finally washed out quantitatively on to the filter.

The liquid contents of flask F are then filtered through the funnel, the mycelium being retained in the flask. It is carefully washed about ten times with separate 10–20 c.c. quantities of hot distilled water, the flask being heated on the boiling water bath for a short time after each successive addition of water. The mycelium is finally washed out into the Buchner funnel, drained dry, and the combined filtrate and washings cooled and made up to 500 c.c. for analysis.

The Buchner funnel and contents are then placed in the oven at 100° C. till the

mycelium is completely dry. The mycelium and most of the kieselguhr are then carefully peeled off the filter paper, weighed in a closed weighing bottle, and ground up in a mortar until a perfectly even sample is obtained. This is again dried and its carbon content determined by wet combustion of a portion. The weight of kieselguhr left adhering to the filter paper is estimated by ashing the latter. From the figures thus obtained the dry weight of mycelium, the total carbon (2), and therefore the percentage of carbon in the mycelium is found.

(3) *Carbon as compounds in solution.*—The total carbon present as compounds in solution (3) has been divided for the purposes of analysis into seven sub-classes:—

- (3a) Carbon as residual glucose.
- (3b) Carbon as carbon dioxide in solution.
- (3c) Carbon as volatile acids.
- (3d) Carbon as non-volatile acids.
- (3e) Carbon as volatile neutral compounds.
- (3f) Carbon as non-volatile neutral compounds (including synthetic compounds).
- (3g) Carbon unaccounted for.

Hence the sum of (3a), (3b), (3c), (3d), (3e), (3f), (3g) is equal to (3), *i.e.*, the total carbon in solution.

The methods used for the separation and analysis of each sub-class will now be described in detail.

(3) *Total carbon as compounds in solution.*—The carbon content of duplicate portions of 10 c.c. of the filtrate from the mycelium (p. 19) is estimated in the usual way by wet combustion.

Here it may be pointed out that a very useful check on the experimental details up to this point is afforded by the fact that the sum of the carbon present in each of the four classes—

- (a) Carbon in sulphuric bubbler (1a),
- (b) Carbon in carbon dioxide (1b),
- (c) Carbon in mycelium (2),
- (d) Carbon in compounds in solution (3),

should equal the total carbon present in the culture medium, at the commencement of the metabolism experiment, a figure which is determined for each batch of medium made up. Any appreciable discrepancy points either to a leak of carbon dioxide through faulty joints in the apparatus or to errors in the quantitative estimations. Although almost three hundred complete metabolism experiments on different types of fungi have been carried out in this laboratory, it has been found that the sum of the above four classes accounts for 96–100 per cent. of the carbon initially supplied, in at least 95 per cent. of the experiments.

(3a) *Carbon as residual glucose*.—Since the carbon as residual glucose often amounts to the greater part of the carbon in solution, it is essential that the sugar should be determined with as great accuracy as possible. The method adopted for the estimation of glucose, and used in the preparation of carbon balance sheets, is that described by SHAFFER and HARTMANN (1921). This method involves copper reduction and the oxidation of the cuprous oxide by iodine in *acid* solution.

It was feared that the method might not give accurate results, because of the absorption of iodine, in those cases where unsaturated metabolism products are present. This fear seems groundless, however, since in a test on a solution of glucose containing fumaric acid the divergences from the calculated results were all within the limits of experimental error.

A check was, however, kept on the results obtained by the above method by carrying out additional estimations of the glucose by each of three other methods of different types whenever sufficient material was available. Further, these methods occasionally afforded valuable information on the types of metabolism products formed, in those cases where discrepancies were observed between the results obtained by them and by the SHAFFER and HARTMANN method. The three methods used were :—

(a) *Polarimeter*.—The glucose was estimated in the usual way by measuring the optical rotation of the solution in the polarimeter. In several cases, which are referred to in more detail in Parts III, IV and VI, there were very marked discrepancies between the figures for glucose as estimated by the SHAFFER and HARTMANN method, and as calculated from the optical rotation. These discrepancies are caused by the production of optically active substances which have no reducing effect on copper solutions, a notable example being gluconic acid.

(b) *WOOD-OST method* (1904).—This well-known method, involving copper reduction like the SHAFFER and HARTMANN method, invariably gave results agreeing with those obtained in the latter method, except in cases where traceable mistakes had been made.

(c) *HINTON and MACARA method* (1924).—The principle involved in this method is the oxidation of the glucose by iodine in *alkaline* solution. It is a very simple method, giving accurate results with pure glucose. However, in the presence of any substance giving rise to iodoform in *alkaline* iodine solution, *e.g.*, alcohol, acetaldehyde, acetone, kojic acid, or any other substance absorbing iodine, values are obtained which are sometimes far in excess of those obtained in the SHAFFER and HARTMANN method. This fact proved of considerable use in investigating the products of metabolism of some of the fungi.

(3b) *Carbon as carbon dioxide in solution*.—The carbon present in the metabolism solution as carbon dioxide, whether as the free gas or in combination as carbonates, is estimated by the VAN SLYKE method, originally described for the estimation of carbon dioxide in blood (1917). The method consists in removing the carbon dioxide *in vacuo*, in a special apparatus designed by VAN SLYKE, from the acidified liquid and

measuring its volume. Triplicate estimations are carried out on 1 c.c. quantities, and the weight of carbon calculated from the volume of carbon dioxide measured.

(3c) *Carbon as volatile acids*.—Included in this class are all the organic acids which are volatile in steam, including all the lower fatty acids up to, say, valeric acid. In order to isolate and analyse the volatile acids the latter are separated by distillation *in vacuo* of the metabolism liquid, after acidification with phosphoric acid. The acids are absorbed in a measured amount of standard baryta, the excess of baryta is titrated, a correction made for carbon dioxide, and the acidity due to volatile acids is calculated. The solution of the barium salts of the volatile acids is evaporated to dryness, weighed, and the carbon content determined by wet combustion.

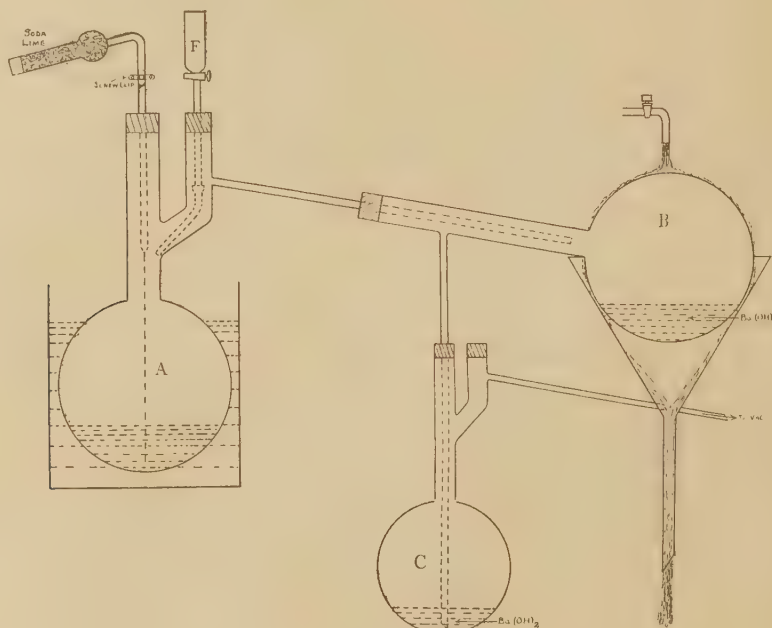


FIG. 3.

The details of the method are as follows: 100 c.c. of the metabolism solution [or, in case of scarcity, the residue from the estimation of volatile neutral matter (3e)] are introduced into the litre Claisen distilling flask A, forming part of the vacuum distillation apparatus shown in fig. 3. 20 c.c. of standard N/4 baryta are run from a burette into the flasks B and C—15 c.c. into B and 5 c.c. into C—and washed in with CO₂-free distilled water. 10 c.c. of 10 per cent. phosphoric acid are then added to flask A, the apparatus connected up, evacuated, and the liquid in flask A evaporated to about 20 c.c., the volatile acids being absorbed in the baryta. 150 c.c. of CO₂-free water are run into A through the funnel F, without letting in air, and the distillation is repeated. Three further amounts of 150 c.c. of CO₂-free water are added and distilled, the liquid

being evaporated to about 20 c.c. each time. Under these conditions the volatile acids are completely separated. The baryta from B and C is washed out into a litre conical flask and titrated with N/10 sulphuric acid to neutrality with phenolphthalein. In order to avoid errors in the subsequent combustion of the barium salts, a known amount—25 c.c.—of aqueous phenolphthalein is used as an indicator and a correction is made in the weight of the barium salts, and in their carbon content, for the weight of phenolphthalein in 25 c.c. and for its carbon content. Errors due to carbon dioxide are corrected by rinsing out flasks B and C with 10 c.c. of N/10 H_2SO_4 , washing this into the litre conical flask, and boiling under reflux to remove carbon dioxide. After cooling, the excess of sulphuric acid is titrated back with baryta and the carbon dioxide calculated. The solution is then filtered from the precipitate of barium sulphate, and the filtrate evaporated to dryness. The residue of barium salts of the volatile acids is weighed and its carbon content determined by wet combustion.

From the weight of the barium salts of the volatile acids, their titration value, and their carbon content, it is possible to obtain an approximate idea as to their composition.

(3d) *Carbon as non-volatile acids.*—The problem of the separation of the non-volatile acids proved to be difficult of solution. A reasonably satisfactory method was obtained, however, by taking advantage of the fact that, while the calcium salts of the volatile acids are relatively soluble in alcohol, those of the non-volatile acids are practically insoluble. The experimental basis for this was obtained by determining the solubilities in 80 per cent. alcohol of the calcium salts of a number of acids likely to be met with in fermentation processes. This was carried out by adding to 20 c.c. of a saturated aqueous solution of the calcium salt of the acid under investigation 80 c.c. of 97 per cent. alcohol, making a final volume of 100 c.c. The mixture, after standing overnight, was filtered, an aliquot part evaporated to dryness, the residue ignited with strong sulphuric acid and the calcium sulphate weighed. From this the weight of organic acid present originally as the calcium salt was calculated.

The results obtained are given in Table II.

It will be seen that the calcium salts of the non-volatile acids have solubilities in 80 per cent. alcohol so low that they may be regarded as insoluble. On the other hand, the calcium salts of the volatile acids are very soluble, so that separation of the two classes by this means is practicable. There still remains the case of lactic acid, a common product of fermentation, which is not readily volatile and thus would not be accurately estimated under section (3c). It is unfortunately not included in class (3d) either, since its calcium salt is soluble in 80 per cent. alcohol to the extent of 334 mgm. of free lactic acid in 100 c.c. of solution, using the same method of expressing solubilities as in the following table. It is, however, included in class (3g) and is referred to later under that heading.

The details of the method used for the separation and analysis of the non-volatile acids are as follows: 50 c.c. of the metabolism solution are pipetted into a 250 c.c. measuring

TABLE II.—Solubilities of the calcium salts of various organic acids in 80 per cent. alcohol at 15° C.
(The solubilities are expressed as milligrams of free acid per 100 c.c. solution.)

| Acid. | Solubility. |
|----------------------------|-------------|
| <i>Non-volatile acids—</i> | |
| Glycollic | 3.6 |
| Oxalic | 0.3 |
| Fumaric | 2.1 |
| Succinic | 3.0 |
| Tartaric | 2.5 |
| Citric | 6.3 |
| Malic | 19.9 |
| Gluconic | 3.9 |
| <i>Volatile acids—</i> | |
| Formic | 152.3 |
| Acetic | 1,262 |
| Propionic | 2,674 |
| Butyric | 3,485 |
| Pyruvic | 76 |

flask. To this is added about 150 c.c. of 97 per cent. alcohol, and any precipitate formed is filtered off. To the clear solution is then added a little phenol red and ammonia, drop by drop, until a permanent pink colour is obtained. The calcium salts are then precipitated by the addition of 5 c.c. of 20 per cent. calcium acetate solution, more ammonia if necessary, and sufficient alcohol to make up to 250 c.c. The mixture is well shaken and allowed to stand overnight. It is then filtered on a tared asbestos Gooch filter (the asbestos having been previously extracted with hydrochloric acid and ignited), dried and reweighed. This gives the weight of the calcium salts of the non-volatile acids. Their carbon content is then determined by the usual wet combustion method, the pad of asbestos as a whole being transferred to the combustion flask. From the weight of the calcium salts and their carbon content an approximate idea can be got of the composition of the non-volatile acids.

(3e) *Carbon as volatile neutral compounds.*—This class includes such compounds as alcohols, ketones, aldehydes, &c., which are readily separated from volatile acids and other compounds by distillation of the metabolism solution, which, as stated on p. 19, is neutralized to pH 7.0 before analysis.

100 c.c. of the metabolism solution are distilled from a Claisen distillation flask, through a condenser, and the distillate is collected in a measuring flask cooled in ice. If only small amounts of volatile compounds are present, only 50 c.c. of distillate are collected. If, on the other hand, relatively large amounts are present, a second 50 c.c. of distillate are collected, after cooling the distillation flask, adding 50 c.c. of distilled water, and redistilling. The carbon content of the distillate is then estimated in an aliquot portion (5 c.c. or 10 c.c.) in the usual apparatus.

(3f) *Non-volatile neutral compounds. Synthetic substances.*—No satisfactory method has been evolved for the separation and estimation of all the non-volatile neutral compounds which would be likely to occur in a fermentation solution. This class covers a wide range of compounds of a number of types, including synthetic compounds (proteins, peptones, &c.), polyhydric alcohols and polyhydric neutral compounds generally. It was therefore necessary to estimate the synthetic compounds separately, leaving the rest of the non-volatile neutral compounds to be included in class (3g), to be estimated separately later, should occasion arise (*cf.* mannitol in Part X and kojic acid in Part VIII).

The synthetic substances, including proteins, peptones, &c., are separated from the metabolism solution by precipitation with colloidal iron solution, and estimated as follows:—To 100 c.c. of the metabolism solution, 25 c.c. of colloidal iron solution are added drop by drop, the flask being shaken vigorously during the addition. The flask is heated in a boiling water bath to flocculate the iron precipitate which carries down with it any proteins, peptones, &c., present. The precipitate is then filtered on a kieselguhr filter, prepared as described on page 19, washed, dried, peeled from the filter paper and the mixture of ferric hydroxide and kieselguhr analysed in the usual apparatus.

(3g) *Carbon unaccounted for.*—This class, which includes the majority of the non-volatile neutral compounds, including particularly the polyhydric alcohols and other neutral compounds, has proved of particular interest. A comparatively large figure in this class is an indication that the fungus in question is worthy of further experimental investigation, since it indicates the production in appreciable quantities of some, possibly new, fermentation compounds, by the micro-organism under investigation. In interpreting the results obtained, however, care must be taken not to overlook the fact that lactic acid is included with the non-volatile neutral compounds (see page 23).

The figure "Carbon unaccounted for" is, of course, not an experimental one, but is obtained by subtracting from the total carbon in solution (3) the sum of the carbon in the sub-classes (3a), (3b), (3c), (3d), (3e) and (3f).

In concluding this section it should be said that it is necessary to keep a strict check on all reagents used during the analysis, and blank estimations were therefore made on all samples of chromic and sulphuric acids used in the wet combustion method, corrections being made later for the carbon found. Particular care is necessary with the chromic acid, some samples of which give large carbon blanks. Complete analyses have also been carried out on different batches of media, treating the unsown CZAPEK-DOX solution as a metabolism solution and carrying through a complete analysis on each flask so chosen. The averages of these figures for the classes (3a) to (3f), which were in no case very high, were used to correct the final carbon balance sheets, all of which are thus made up from figures corrected for blanks of reagents used, and for blanks of methods.

Large numbers of these balance sheets were prepared and will be found in Parts III, IV, V and VI of this series.

Summary.

A description is given of the methods elaborated for the quantitative determination of different *types* of metabolic compounds formed by the action of micro-organisms on carbohydrates. From these figures a carbon balance sheet is prepared for the micro-organism under investigation.

The methods adopted are described under the following heads :—

- (A) The estimation of carbon by wet combustion (p. 11).
 - (B) Apparatus for metabolism experiments (p. 14).
 - (C) Technique employed for the cultivation of micro-organisms in the metabolism experiments (p. 15).
 - (D) Methods of separation and analysis of the metabolic products (p. 17).
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Studies in the Biochemistry of Micro-organisms.

PART III.—*Quantitative examination by the carbon balance sheet method of the types of products formed from glucose by species of Aspergillus.*

By JOHN HOWARD BIRKINSHAW, JOHN HENRY VICTOR CHARLES, HAROLD RAISTRICK
and JOHN ALEXANDER ROBERTSON STOYLE.

The first large group of fungi chosen for investigation by the methods described in Part II was the *Aspergillus* group. The *Aspergilli* were chosen primarily because they are relatively easy to grow on synthetic media and because a wide variety of cultures was available in different parts of the world.

Throughout this work conditions have been kept as nearly standard as possible so that results obtained at any time during the progress of the work should be comparable. With this end in view all the metabolism experiments were carried out at one temperature, namely, 23–25° C. One medium, consisting of a CZAPEK-DOX medium of the composition given in Part I, p. 7, was used throughout. This medium was made up in large amounts and pipetted out accurately, in 250 c.c. quantities, into a number of 750 c.c. conical flasks.

Particular care was taken to ensure that all cultures had uniform aeration. In the course of this work aeration has been shown repeatedly to have a fundamental effect on the type and yield of end-product given by any particular species. Hence each culture was aerated every day by passing 500 c.c. of sterile air over the surface of the mycelium, during the space of about an hour. For the remainder of the 24 hours the whole system of flask, absorption bubblers, &c., was closed. It is emphasized that the products and yields described are true for the moulds in question only when they receive the amount of air mentioned, *i.e.*, a *restricted air supply*. It has been shown repeatedly that the products arising when the mould receives an *unrestricted air supply* are often quite different both in nature and quantity.

The general arrangements of the experiments were those described in Part II on pp. 15 to 17. It is worthy of note that, since in each experiment 250 c.c. of 5 per cent. glucose solution is employed (*i.e.*, 12.5 gm. of glucose or 5 gm. of carbon as glucose), an approximate estimate may be obtained of the percentage yield of any type of carbon end-product by multiplying the figure given in the balance sheet by 20.

The results obtained are given in the following pages and are arranged in groups according to the classification of the *Aspergilli* adopted in their recent book 'The *Aspergilli*,' by C. THOM and MARGARET B. CHURCH (Williams & Wilkins Company, Baltimore, 1926). It is interesting to note that, with very few exceptions

the biochemical characteristics of any particular group, as deduced from the carbon balance sheets, place the different species of *Aspergillus* in the groups assigned to them by THOM and CHURCH. The catalogue numbers, *e.g.*, Ac. 65, are those used in the Ardeer catalogue of fungi.

Group 1.—*A. clavatus* group and associated species.

The history of the cultures used for investigation which are included in this group is the following :—

- (1) *A. clavatus* DESMAZIÈRES, Catalogue No. Ac. 65. Obtained from PRIBŮRÁM, Vienna.
- (2) *A. clavatus*, Catalogue No. Ac. 86. Obtained from Miss CHURCH and bore the number 107.
- (3) *A. clavatus*, Catalogue No. Ac. 95. Obtained from the British National Collection of Type Cultures and bore their number 978. It also bore the number Washington 107 and is probably identical with the strain Ac. 86.
- (4) *A. clavatus*, Catalogue No. Ac. 87. Obtained from Miss CHURCH and bore the THOM and CHURCH No. 138. This culture, although received as *A. clavatus*, is probably *A. giganteus* WEHMER, referred to by THOM and CHURCH in their book on p. 100 as culture number 138.
- (5) *A. giganteus* WEHMER, Catalogue No. Ac. 32. Purchased from Centraalbureau voor Schimmelcultures at Baarn.

The collected balance sheets are given in Table I.

Consideration of the results given in Table I shows that all the five species included in this group give balance sheets of the same type and having the following characteristics: (1) All give rise to large amounts of volatile neutral compounds (alcohol) and hence have high respiration coefficients. (2) None of them has any other outstanding characteristics, as they all give only moderate amounts of acid, whether volatile or non-volatile, and, with the exception of Ac. 87, all give only moderate amounts of "carbon unaccounted for."

The main biochemical characteristic of the *A. clavatus* group thus appears to be the production of large amounts of alcohol from glucose.

Group 2.—The *A. glaucus* group.

The species included in Group 2, which forms a well-defined group having marked biochemical characteristics, are the following :—

- (1) *A. glaucus* LINK, Catalogue No. Ac. 36. Purchased from Baarn.
- (2) *A. ferrugineus* FÜCKEL, Catalogue No. Ac. 39. Purchased from Baarn.
- (3) *A. medius* MEISSNER, Catalogue No. Ac. 45. This is the culture 4724.45 referred to on p. 108 of THOM and CHURCH's book, and was purchased from Baarn.

TABLE I.—Carbon balance sheets for *A. clavatus* group and associated species of *Aspergillus*.

| Species of <i>Aspergillus</i> : | <i>A. clavatus</i> DESMAZIÈRES. | | | | <i>A. giganteus</i> WEHMER. | |
|---|------------------------------------|--------|--------|--------|--------------------------------|--------|
| | Ac. 65. | Ac. 86 | Ac. 95 | Ac. 87 | Ac. 32 | Ac. 32 |
| Catalogue number : | 94 | 121 | 136 | 122 | 42 | 66 |
| Experiment number : | 27 | 41 | 26 | 46 | 21 | 34 |
| Incubation period in days : | | | | | | |
| <i>Carbon Balance Sheet.</i> | | | | | | |
| Carbon in solution (start) ... gm. | 4.834 | 4.901 | 5.043 | 4.901 | 5.094 | 5.053 |
| Carbon in H ₂ SO ₄ | 0.015 | 0.022 | 0.021 | 0.008 | 0.007 | 0.006 |
| " in CO ₂ | 1.692 | 1.913 | 1.777 | 1.644 | 1.171 | 1.198 |
| " in mycelium | 0.359 | 0.349 | 0.462 | 0.454 | 0.630 | 0.420 |
| " in solution (end) | 2.684 | 2.512 | 2.596 | 2.714 | 3.147 | 3.342 |
| " accounted for | 4.750 | 4.796 | 4.856 | 4.820 | 4.955 | 4.966 |
| " accounted for ... per cent. | 98.3 | 97.8 | 96.3 | 98.3 | 97.3 | 98.3 |
| <i>Analysis of Solution.</i> | | | | | | |
| Carbon in residual glucose ... gm. | 0.554 | 0.009 | 0.518 | 0.915 | 1.768 | 2.144 |
| " in CO ₂ in solution | 0.013 | 0.007 | 0.007 | 0.011 | 0.022 | 0.009 |
| " in volatile acids | 0.019 | 0.009 | 0.003 | 0.007 | 0.022 | 0.015 |
| " in non-volatile acids | 0.108 | 0.089 | 0.106 | 0.124 | 0.059 | 0.065 |
| " in volatile neutral compounds .. | 1.629 | 2.064 | 1.553 | 0.835 | 0.807 | 0.594 |
| " in synthetic compounds .. | 0.078 | 0.151 | 0.150 | 0.125 | 0.054 | 0.037 |
| Total carbon accounted for | 2.401 | 2.329 | 2.337 | 2.017 | 2.732 | 2.864 |
| " " in solution | 2.684 | 2.512 | 2.596 | 2.714 | 3.147 | 3.342 |
| Carbon unaccounted for (by difference),, | 0.283 | 0.183 | 0.259 | 0.697 | 0.415 | 0.478 |
| <i>Residual Glucose.</i> | | | | | | |
| Glucose (by polarimeter) ... per cent. | 0.292 | 0.004 | 0.308 | 0.590 | 1.044 | 1.092 |
| " (SHAFFER-HARTMANN) .. | 0.277 | 0.005 | 0.259 | 0.457 | 0.884 | 1.072 |
| " (WOOD-OST) | 0.280 | — | — | 0.480 | — | 1.072 |
| " (by alkaline iodine) .. | 0.321 | 0.087 | 0.323 | 0.559 | — | — |
| <i>Acids.</i> | | | | | | |
| Titration (N/1 acid) c.c. | 3.0 | 2.6 | 3.6 | 1.7 | 0.3 | 0.7 |
| Volatile acids (N/1 acid) | 1.22 | 1.23 | 1.62 | 0.73 | 1.85 | 0.57 |
| Barium salts (weight) gm. | 0.067 | 0.134 | 0.051 | 0.044 | — | 0.045 |
| Calcium salts (weight) | 0.358 | 0.332 | 0.662 | 0.535 | 0.568 | 0.348 |
| Volume of oxygen absorbed ... c.c. | 1405 | 1445 | 1915 | 1900 | 1213 | 1514 |
| Respiration coefficient | 2.27 | 2.48 | 1.74 | 1.63 | 1.84 | 1.49 |
| Mycelium (weight) gm. | 0.736 | 0.649 | 0.874 | 0.884 | 1.318 | 0.817 |
| " (carbon) per cent. | 48.8 | 53.9 | 52.9 | 51.4 | 47.8 | 51.3 |

- (4) *A. mollis* BAINIER and SARTORY, Catalogue No. Ac. 58. Purchased from Baarn.
- (5) *A. Scheelei* BAINIER and SARTORY, Catalogue No. Ac. 60. Purchased from Baarn.
- (6) *A. disjunctus* BAINIER and SARTORY, Catalogue No. Ac. 61. Purchased from Baarn.
- (7) *A. novus* WEHMER, Catalogue No. Ac. 46. Purchased from PRIBŘAM. This culture is referred to in THOM and CHURCH's book on p. 206 as number 4724.46.
- (8) *A. repens*, Catalogue No. Ac. 5. This species was isolated at Ardeer from a diseased Larch twig and was diagnosed by Miss CHURCH as a member of the *A. repens-glaucus* group.
- (9) *A. repens*, Catalogue No. Ac. 6. This species was isolated at Ardeer from mouldy rhubarb jam and was also diagnosed by Miss CHURCH as belonging to this group.

The carbon balance sheets are collected in Table II.

The *Aspergillus glaucus* group is a well-defined group of organisms having very marked biochemical characteristics, which distinguish it from any of the other groups described in this paper. This is evidenced by the following observations :—

(1) None of the cultures grows well on the CZAPEK-Dox medium used, as is indicated by the extreme length of the incubation period necessary for the conversion of a relatively small amount of glucose (see figures for incubation period in days). This is also evidenced by the relatively small weights of mycelium produced during this period.

(2) All these species give rise to dark-coloured metabolism solutions showing marked fluorescence. Thus with *A. ferrugineus*, Ac. 39, the filtered metabolism solution was of a dark brownish-red colour with a most strikingly marked greenish fluorescence. Similar remarks apply to *A. medius*, Ac. 45, and to a lesser extent to the other species.

(3) In marked contrast to Group 1, none of the species in Group 2 gives rise to any volatile neutral compounds. This consideration confirms our opinion that the culture received by us from PRIBŘAM and called *A. novus* WEHMER should be included in this group. A sub-culture was sent to THOM and CHURCH, who refer to it on p. 206 of their book and place it in the *A. flavus-oryzæ* group. Consideration of the results given in Table XIII shows that all members of the *A. flavus-oryzæ* group give rise to large amounts of volatile neutral compounds, and in addition almost all give kojic acid. *A. novus* WEHMER is entirely lacking in either of these characteristics and its balance sheet is entirely of the type of Group 2. The fact that none of the species included in this group produces volatile neutral compounds is further supported by the fact that all of them have respiration coefficients approximating to unity.

(4) The main positive biochemical characteristics of this group are :—

- (a) The production of relatively large amounts of "carbon unaccounted for."
- (b) The production of a medium amount of non-volatile acids.
- (c) The production of some optically active compound, as is indicated by the fact

TABLE II.—Carbon balance sheets for *A. glaucus* group.

| Species of <i>Aspergillus</i> : | <i>A. glaucus</i> LINK. | | <i>A. ferrugineus</i> FÜCKEL. | <i>A. medius</i> MEISSNER. | <i>A. mollis</i> BAINIER and SARTORY. | <i>A. Scheelei</i> BAINIER and SARTORY. | <i>A. dis-junctus</i> BAINIER and SARTORY. | <i>A. novus</i> WEHMER. | <i>A. repens</i> . | |
|--|-------------------------|--------|-------------------------------|----------------------------|---------------------------------------|---|--|-------------------------|--------------------|-------|
| | Ac. 36 | Ac. 36 | Ac. 39 | Ac. 45 | Ac. 58 | Ac. 60 | Ac. 61 | Ac. 46 | Ac. 5 | Ac. 6 |
| Catalogue number : | 50 | 72 | 58 | 59 | 80 | 97 | 100 | 61 | 44 | 47 |
| Experiment number : | 52 | 78 | 34 | 52 | 68 | 55 | 70 | 59 | 59 | 58 |
| Incubation period in days : | | | | | | | | | | |
| <i>Carbon Balance Sheet.</i> | | | | | | | | | | |
| Carbon in solution (start) ... gm. | 5.094 | 5.053 | 5.094 | 5.094 | 4.834 | 4.834 | 4.834 | 5.094 | 5.094 | 5.094 |
| Carbon in H ₂ SO ₄ ... " | 0.001 | nil | 0.001 | nil | nil | 0.001 | nil | nil | nil | 0.001 |
| " in CO ₂ ... " | 1.190 | 0.947 | 0.574 | 0.903 | 0.846 | 1.121 | 1.196 | 0.748 | 0.752 | 0.512 |
| " in mycelium ... " | 0.393 | 0.255 | 0.181 | 0.323 | 0.318 | 0.379 | 0.444 | 0.186 | 0.314 | 0.209 |
| " in solution (end) ... " | 3.453 | 3.738 | 4.248 | 3.826 | 3.583 | 3.259 | 3.078 | 4.048 | 3.949 | 4.198 |
| " accounted for ... " | 5.037 | 4.940 | 5.004 | 5.052 | 4.747 | 4.760 | 4.718 | 4.982 | 5.015 | 4.920 |
| " accounted for ... per cent. | 98.9 | 97.8 | 98.2 | 99.2 | 98.2 | 98.5 | 97.6 | 97.8 | 98.5 | 96.6 |
| <i>Analysis of Solution.</i> | | | | | | | | | | |
| Carbon in residual glucose ... gm. | 2.388 | 2.536 | 3.772 | 3.072 | 2.592 | 1.980 | 1.786 | 2.970 | 3.082 | 3.792 |
| " in CO ₂ in solution ... " | 0.009 | 0.004 | 0.003 | nil | 0.002 | 0.001 | 0.005 | 0.003 | nil | 0.020 |
| " in volatile acids ... " | 0.001 | nil | 0.004 | nil | nil | nil | nil | 0.001 | nil | 0.001 |
| " in non-volatile acids ... " | 0.275 | 0.384 | 0.119 | 0.254 | 0.197 | 0.469 | 0.268 | 0.249 | 0.219 | 0.174 |
| " in volatile neutral compounds ... " | 0.009 | 0.001 | 0.001 | 0.004 | 0.003 | nil | nil | 0.010 | 0.007 | 0.022 |
| " in synthetic compounds ... " | 0.082 | 0.097 | 0.069 | 0.146 | 0.063 | 0.057 | 0.071 | 0.119 | 0.082 | 0.054 |
| Total carbon accounted for ... " | 2.764 | 3.022 | 3.968 | 3.476 | 2.857 | 2.507 | 2.130 | 3.352 | 3.390 | 4.063 |
| " " in solution ... " | 3.453 | 3.738 | 4.248 | 3.826 | 3.583 | 3.259 | 3.078 | 4.048 | 3.949 | 4.198 |
| Carbon unaccounted for (by difference) " | 0.689 | 0.716 | 0.280 | 0.350 | 0.726 | 0.752 | 0.948 | 0.696 | 0.559 | 0.135 |
| <i>Residual Glucose.</i> | | | | | | | | | | |
| Glucose (by polarimeter) ... per cent. | 1.229 | 1.446 | 1.858 | 1.440 | 1.451 | 1.263 | 1.103 | 1.615 | 1.721 | 2.170 |
| " (SHAFFER-HARTMANN) ... " | 1.194 | 1.268 | 1.886 | 1.536 | 1.296 | 0.990 | 0.893 | 1.485 | 1.541 | 1.896 |
| " (WOOD-OSR) ... " | — | 1.267 | 1.776 | 1.483 | 1.287 | 1.000 | 0.890 | 1.435 | — | — |
| " (by alkaline iodine) ... " | — | 1.263 | — | — | 1.299 | 1.034 | 0.917 | — | — | — |
| <i>Acids.</i> | | | | | | | | | | |
| Titration (N/1 acid) ... c.c. | 2.2 | 5.7 | 0.6 | 2.0 | 3.7 | 5.5 | 4.8 | 4.3 | 3.3 | 3.6 |
| Volatile acids (N/1 acid) ... " | 0.26 | 0.07 | 0.38 | nil | nil | nil | nil | nil | 1.29 | 0.61 |
| Barium salts (weight) ... gm. | 0.009 | 0.010 | 0.061 | 0.001 | 0.001 | 0.014 | 0.005 | 0.043 | 0.014 | 0.031 |
| Calcium salts (weight)... " | 1.499 | 1.160 | 0.485 | 0.894 | 0.742 | 1.201 | 0.978 | 0.964 | 0.737 | 2.263 |
| Volume of oxygen absorbed ... c.c. | 2191 | 1986 | 1067 | 1701 | 1613 | 1911 | 2075 | 1358 | 1539 | 914 |
| Respiration coefficient ... " | 1.02 | 0.89 | 1.01 | 0.99 | 0.98 | 1.09 | 1.08 | 1.03 | 0.91 | 1.09 |
| Mycelium (weight) ... gm. | 0.696 | 0.442 | 0.307 | 0.602 | 0.575 | 0.681 | 0.824 | 0.335 | 0.561 | 0.411 |
| " (carbon) ... per cent. | 56.5 | 57.6 | 59.1 | 53.7 | 55.3 | 55.7 | 53.9 | 55.5 | 56.0 | 50.9 |

that the glucose content determined by polarimeter is considerably higher than that given by the copper reduction methods.

(d) Uniformly high percentage of carbon in the mycelium.

(5) Practically all the species show an entire absence of the production of any volatile acids.

To sum up, the *Aspergillus glaucus* group is of particular biochemical interest since the species included in it have a well-marked type of metabolism, as a result of which they give rise to considerable yields of compounds designated here "carbon unaccounted for," the nature of which has not yet been investigated.

Group 3.—Intermediate Forms.

Only two species are included in this group :—

- (1) *A. minimus* WEHMER, Catalogue No. Ac. 68. Purchased from PRIBĚM.
- (2) *A. conicus* BLOCHWITZ, Catalogue No. Ac. 41. Purchased from Baarn.

The balance sheets for these species are given in Table III. They are entirely different in type, and indicate that the two species are not closely related biochemically. *A. minimus* appears to be related in its biochemical characteristics most closely to the *Aspergillus glaucus* group, while *A. conicus* does not appear to have any outstanding characteristic which correlates it with any other group.

Group 4.—The *A. fumigatus* group.

Four species of *Aspergillus* falling in this group were examined :—

- (1) *A. fumigatus* FRESENIUS, Catalogue No. Ac. 15. Purchased from the British National Collection of Type Cultures and bore their Catalogue No. 367.
- (2) *A. fumigatus*, Catalogue No. Ac. 71. Purchased from the British National Collection of Type Cultures and bore their Catalogue No. 982.
- (3) *A. fumigatus* (ascosporic), Catalogue No. Ac. 70. Purchased from the British National Collection of Type Cultures and bore their Catalogue No. 1326.
- (4) *A. Fischeri* WEHMER, Catalogue No. Ac. 38. Purchased from Baarn.

The carbon balance sheets for these species are given in Table IV. From a biochemical point of view they are not very interesting, since the species in this group do not appear to give rise to appreciable amounts of any end-products from glucose other than CO₂. They all grow well on CZAPEK-DOX medium with the possible exception of *A. Fischeri*. They appear to be related—in the sense that they do not give rise to any end-products—to the *A. versicolor*–*A. Sydowi* group described on p. 35.

TABLE III.—Carbon balance sheets for intermediate forms of *Aspergillus*.

| Species of <i>Aspergillus</i> : | <i>A. minimus</i> WEHMER. | | <i>A. conicus</i> BLOCHWITZ. |
|---|---------------------------|--------|---------------------------------|
| Catalogue number : | Ac. 68 | Ac. 68 | Ac. 41 |
| Experiment number : | 101 | 110 | 53 |
| Incubation period in days : | 45 | 72 | 46 |
| <i>Carbon Balance Sheet.</i> | | | |
| Carbon in solution (start) gm. | 4.834 | 5.001 | 5.094 |
| Carbon in H ₂ SO ₄ " | 0.001 | nil | 0.001 |
| " in CO ₂ " | 1.209 | 1.572 | 0.381 |
| " in mycelium " | 0.782 | 1.144 | 0.260 |
| " in solution (end) " | 2.758 | 2.136 | 4.337 |
| " accounted for " | 4.750 | 4.852 | 4.979 |
| " accounted for per cent. | 98.5 | 97.0 | 97.8 |
| <i>Analysis of Solution.</i> | | | |
| Carbon in residual glucose gm. | 1.690 | 1.192 | 3.850 |
| " in CO ₂ in solution " | 0.001 | 0.003 | 0.009 |
| " in volatile acids " | nil | nil | 0.010 |
| " in non-volatile acids " | 0.224 | 0.251 | 0.111 |
| " in volatile neutral compounds " | 0.005 | 0.002 | 0.104 |
| " in synthetic compounds " | 0.047 | 0.073 | 0.051 |
| Total carbon accounted for " | 1.967 | 1.521 | 4.135 |
| " " in solution " | 2.758 | 2.136 | 4.337 |
| Carbon unaccounted for (by difference) " | 0.791 | 0.615 | 0.202 |
| <i>Residual Glucose.</i> | | | |
| Glucose (by polarimeter) per cent. | 1.061 | 0.832 | 2.045 |
| " (SHAFFER-HARTMANN) " | 0.845 | 0.596 | 1.925 |
| " (WOOD-OST)... .. " | 0.928 | 0.600 | — |
| " (by alkaline iodine) " | 0.825 | 0.612 | — |
| <i>Acids.</i> | | | |
| Titration (N/1 acid) c.c. | 3.1 | 3.0 | 0.9 |
| Volatile acids (N/1 acid) " | nil | 0.39 | 1.12 |
| Barium salts (weight) gm. | 0.018 | nil | 0.072 |
| Calcium salts (weight) " | 0.804 | 0.871 | 0.459 |
| Volume of oxygen absorbed c.c. | 1906 | 2552 | 602 |
| Respiration coefficient " | 1.19 | 1.15 | 1.21 |
| Mycelium (weight) gm. | 1.456 | 2.070 | 0.518 |
| " (carbon) per cent. | 53.7 | 55.3 | 50.3 |

TABLE IV.—Carbon balance sheets for *A. fumigatus* group.

| Species of <i>Aspergillus</i> : | <i>A.</i> <i>fumigatus</i> FRESENIUS. | | <i>A.</i> <i>fumigatus</i> (ascosporic) | <i>A. Fischeri</i> WEHMER. | |
|---|--|-----------------------|---|----------------------------|--------|
| | Ac. 15 | Ac. 71 | Ac. 70 | Ac. 38 | Ac. 38 |
| Catalogue number : | 20 | 106 | 105 | 137 | 51 |
| Experiment number : | 21 | 55 | 56 | 107 | 42 |
| Incubation period in days : | 21 | 55 | 56 | 107 | 42 |
| <i>Carbon Balance Sheet.</i> | | | | | |
| Carbon in solution (start) gm. | 5.020 | 4.834 | 4.834 | 5.043 | 5.094 |
| Carbon in H ₂ SO ₄ " | 0.001 | 0.001 | 0.001 | 0.002 | 0.001 |
| " in CO ₂ " | 1.115 | 1.342 | 1.186 | 1.819 | 0.436 |
| " in mycelium " | 0.803 | 0.842 | 0.525 | 1.005 | 0.255 |
| " in solution (end) " | 3.108 | 2.536 | 3.055 | 2.012 | 4.318 |
| " accounted for " | 5.027 | 4.721 | 4.767 | 4.838 | 5.010 |
| " accounted for per cent. | 100.2 | 97.9 | 98.6 | 95.9 | 98.3 |
| <i>Analysis of Solution.</i> | | | | | |
| Carbon in residual glucose gm. | 2.656 | 2.024 | 2.708 | 1.325 | 3.920 |
| " in CO ₂ in solution " | 0.010 | 0.014 | 0.008 | 0.002 | 0.010 |
| " in volatile acids " | 0.004 | nil | nil | 0.017 | 0.031 |
| " in non-volatile acids " | 0.069 | 0.080 | 0.118 | 0.131 | 0.081 |
| " in volatile neutral compounds " | 0.007 | 0.008 | nil | 0.039 | 0.022 |
| " in synthetic compounds " | 0.092 | 0.063 | 0.076 | 0.097 | 0.026 |
| Total carbon accounted for " | 2.838 | 2.189 | 2.910 | 1.611 | 4.090 |
| " in solution " | 3.108 | 2.536 | 3.055 | 2.012 | 4.318 |
| Carbon unaccounted for (by difference) " | 0.270 | 0.347 | 0.145 | 0.401 | 0.228 |
| <i>Residual Glucose.</i> | | | | | |
| Glucose (by polarimeter) per cent. | 1.290 | 1.002 | 1.476 | 0.661 | 2.055 |
| " (SHAFFER-HARTMANN) " | 1.328 | 1.012 | 1.354 | 0.663 | 1.960 |
| " (WOOD-OST) " | — | 1.084 | 1.363 | 0.683 | — |
| " (by alkaline iodine) " | — | 1.081 | 1.377 | 0.736 | — |
| <i>Acids.</i> | | | | | |
| Titration (N/1 acid) c.c. | 1.3 | Decrease of 0.7 | 0.4 | 0.6 | 0.6 |
| Volatile acids (N/1 acid) " | 0.09 | nil | nil | 0.67 | 2.20 |
| Barium salts (weight) gm. | — | 0.016 | 0.015 | 0.050 | 0.205 |
| Calcium salts (weight) " | 0.577 | 0.248 | 0.558 | 0.406 | 0.442 |
| Volume of oxygen absorbed c.c. | 1820 | 2266 | 2009 | 2898 | 810 |
| Respiration coefficient " | 1.15 | 1.12 | 1.11 | 1.17 | 1.03 |
| Mycelium (weight) gm. | 1.473 | 1.733 | 0.965 | 1.984 | 0.489 |
| " (carbon) per cent. | 54.5 | 48.6 | 54.4 | 50.6 | 52.0 |

Group 5.—The *A. nidulans* group.

The following seven different strains of *A. nidulans* were examined :—

- (1) *A. nidulans* EIDAM, Catalogue No. Ac. 67. Purchased from Baarn.
- (2) *A. nidulans*, Catalogue No. Ac. 78. Purchased from PRIBRAM and as received bore the label *A. nidulans* (HANN).
- (3) *A. nidulans* var. *Nicollei* PINOY, Catalogue No. Ac. 85. Purchased from Baarn.
- (4) *A. nidulans*, Catalogue No. Ac. 98. Received from Mr. F. T. BROOKS of Cambridge and identified by Miss CHURCH as *A. nidulans*.
- (5) *A. nidulans* (ascosporic), Catalogue No. Ac. 79. Sent by Dr. THOM and Miss CHURCH and bore their Catalogue No. 110. (See THOM and CHURCH's book, p. 139.)
- (6) *A. nidulans*, Catalogue No. Ac. 80. Received from Dr. THOM and Miss CHURCH and bore their Catalogue No. 4882.27.
- (7) *A. nidulans*, Catalogue No. Ac. 9. Isolated at Ardeer and identified as *A. nidulans* by Miss CHURCH.

The carbon balance sheets for these species are given in Table V. Unlike the species in the separate groups so far examined, which have all behaved similarly amongst themselves, there is a sharp difference in biochemical behaviour between the different species of *A. nidulans*. This is limited to one biochemical characteristic, namely the production of volatile neutral compounds (alcohol). Thus, while cultures Ac. 67, 78 and 85 produce considerable amounts of volatile neutral compounds and have respiration coefficients of about 1.6, cultures Ac. 98, 79, 80 and 9 do not produce any alcohol. The different cultures seem to be similar in other respects. Thus, almost all of them give rise to considerable amounts of "carbon unaccounted for," and this has been shown to be almost entirely mannitol, both in the case of those species giving alcohol and in the case of those which did not give it. (These results are given in Part IX.) In addition, all the different strains grow well on the medium used, and none of them gives rise to appreciable amounts of titratable acidity. In fact, three strains, Ac. 79, Ac. 80 and Ac. 9, actually bring about a reduction in the initial acidity of the medium. All strains give rise to amounts of volatile acids which, while small in themselves, are large in comparison with those formed by other groups, *e.g.*, the *A. glaucus* group.

Group 6.—*A. versicolor*—*A. Sydowi* group.

A number of species are included in this group. *A. versicolor* VUILLEMIN has proved to be of common occurrence and a large number of different strains have been collected. Eight strains given in Table VI are representative of the whole :—

- (1) *A. versicolor*, Catalogue No. Ac. 7. Isolated at Ardeer from tobacco and identified by Miss CHURCH.
- (2) *A. versicolor*, Catalogue No. Ac. 8. Isolated at Ardeer from tobacco and identified by Miss CHURCH.

TABLE V.—Carbon balance sheets for *A. nidulans* group.

| Species of <i>Aspergillus</i> : | <i>A. nidulans</i> EIDAM. | <i>A. nidulans</i> | <i>A. nidulans</i> var. <i>Nicollei</i> PINOY. | <i>A. nidulans</i> . | <i>A. nidulans</i> (asco- sporic). | <i>A. nidulans</i> . | <i>A. nidulans</i> . |
|--|----------------------------------|------------------------|---|--------------------------|--|--------------------------|--------------------------|
| Catalogue number : | Ac. 67 | Ac. 78 | Ac. 85 | Ac. 98 | Ac. 79 | Ac. 80 | Ac. 9 |
| Experiment number : | 95 | 111 | 120 | 130 | 112 | 113 | 13 |
| Incubation period in days : | 35 | 44 | 61 | 41 | 40 | 58 | 16 |
| <i>Carbon Balance Sheet.</i> | | | | | | | |
| Carbon in solution (start) ... gm. | 4.834 | 5.001 | 4.901 | 5.043 | 5.001 | 5.001 | 5.020 |
| Carbon in H ₂ SO ₄ ... " | — | 0.011 | 0.012 | nil | 0.002 | nil | 0.002 |
| " in CO ₂ ... " | 1.375 | 1.837 | 1.928 | 1.352 | 1.550 | 1.380 | 0.683 |
| " in mycelium ... " | 0.683 | 0.949 | 0.609 | 1.253 | 1.228 | 0.845 | 0.849 |
| " in solution (end) ... " | 2.663 | 2.064 | 2.207 | 2.273 | 2.143 | 2.728 | 3.577 |
| " accounted for ... " | 4.721 | 4.861 | 4.756 | 4.878 | 4.923 | 4.953 | 5.111 |
| " accounted for ... per cent. | 97.7 | 97.2 | 97.1 | 96.7 | 98.5 | 99.1 | 101.8 |
| <i>Analysis of Solution.</i> | | | | | | | |
| Carbon in residual glucose ... gm. | 1.179 | 0.245 | 0.131 | 1.240 | 1.158 | 2.453 | 3.130 |
| " in CO ₂ in solution ... " | 0.013 | 0.010 | 0.013 | 0.007 | 0.026 | 0.045 | 0.019 |
| " in volatile acids ... " | 0.025 | 0.020 | 0.027 | 0.011 | 0.043 | 0.034 | 0.023 |
| " in non-volatile acids ... " | 0.076 | 0.080 | 0.090 | 0.104 | 0.091 | 0.131 | 0.080 |
| " in volatile neutral compounds ... " | 0.673 | 0.831 | 1.026 | 0.006 | 0.030 | 0.016 | nil |
| " in synthetic compounds ... " | 0.057 | 0.082 | 0.122 | 0.108 | 0.142 | 0.119 | 0.033 |
| Total carbon accounted for ... " | 2.023 | 1.268 | 1.409 | 1.476 | 1.490 | 2.798 | 3.285 |
| " " in solution ... " | 2.663 | 2.064 | 2.207 | 2.273 | 2.143 | 2.728 | 3.577 |
| Carbon unaccounted for (by difference) " | 0.640 | 0.796 | 0.798 | 0.797 | 0.653 | Surplus of 0.070 | 0.292 |
| <i>Residual Glucose.</i> | | | | | | | |
| Glucose (by polarimeter) ... per cent. | 0.696 | 0.251 | 0.128 | 0.736 | 0.820 | 1.254 | 1.550 |
| " (SHAFFER-HARTMANN) ... " | 0.590 | 0.123 | 0.065 | 0.620 | 0.579 | 1.226 | 1.565 |
| " (WOOD-OST) ... " | 0.590 | — | — | 0.620 | 0.583 | 1.212 | — |
| " (by alkaline iodine) ... " | 0.633 | 0.188 | 0.129 | 0.674 | 0.715 | 1.236 | — |
| <i>Acids.</i> | | | | | | | |
| Titration (N/1 acid) ... c.c. | 1.0 | 0.6 | 2.4 | 0.8 | 1.1 | 1.1 | 0.6 |
| Volatile acids (N/1 acid) ... " | 0.89 | 0.34 | 1.50 | 0.84 | 1.37 | 1.41 | 1.55 |
| Barium salts (weight) ... gm. | 0.073 | 0.052 | 0.095 | 0.010 | 0.173 | 0.198 | — |
| Calcium salts (weight) ... " | 0.290 | 0.195 | 0.445 | 0.444 | 0.256 | 0.617 | 0.667 |
| Volume of oxygen absorbed ... c.c. | 1598 | 2114 | 2210 | 1813 | 2295 | 2176 | 970 |
| Respiration coefficient ... | 1.62 | 1.63 | 1.64 | 1.40 | 1.28 | 1.22 | 1.35 |
| Mycelium (weight) ... gm. | 1.379 | 1.920 | 1.209 | 2.314 | 2.391 | 1.554 | 1.830 |
| " (carbon) ... per cent. | 49.5 | 49.4 | 50.4 | 54.2 | 51.4 | 54.3 | 46.4 |

TABLE VI.—Carbon balance sheets for *A. versicolor* group.

| Species of <i>Aspergillus</i> : | | <i>A. versicolor</i> VUILLEMIN. | | | | | | | |
|--|-----------|---------------------------------|-------|-----------------|------------------|--------|--------|--------|--------|
| Catalogue number : | | Ac. 7 | Ac. 8 | Ac. 28 | Ac. 33 | Ac. 47 | Ac. 25 | Ac. 30 | Ac. 18 |
| Experiment number : | | 15 | 40 | 37 | 49 | 109 | 45 | 39 | 98 |
| Incubation period in days : | | 16 | 22 | 15 | 33 | 32 | 36 | 43 | 39 |
| <i>Carbon Balance Sheet.</i> | | | | | | | | | |
| Carbon in solution (start) ... | gm. | 5.020 | 5.094 | 5.094 | 5.094 | 5.001 | 5.094 | 5.094 | 4.834 |
| Carbon in H ₂ SO ₄ ... | " | 0.001 | 0.001 | 0.001 | 0.001 | nil | 0.001 | 0.001 | 0.001 |
| " in CO ₂ ... | " | 1.227 | 1.312 | 1.069 | 0.556 | 0.888 | 0.902 | 0.885 | 0.876 |
| " in mycelium ... | " | 1.155 | 0.945 | 1.002 | 0.497 | 0.580 | 0.598 | 0.550 | 0.397 |
| " in solution (end) ... | " | 2.633 | 2.675 | 2.921 | 3.499 | 3.460 | 3.474 | 3.551 | 3.510 |
| " accounted for ... | " | 5.016 | 4.933 | 4.993 | 5.003 | 4.928 | 4.975 | 4.987 | 4.784 |
| " accounted for ... | per cent. | 99.9 | 96.8 | 98.0 | 98.2 | 98.6 | 97.7 | 97.9 | 98.9 |
| <i>Analysis of Solution.</i> | | | | | | | | | |
| Carbon in residual glucose ... | gm. | 2.030 | 1.948 | 2.256 | 3.778 | 2.884 | 2.769 | 3.104 | 2.992 |
| " in CO ₂ in solution ... | " | 0.025 | 0.032 | 0.016 | 0.012 | 0.028 | 0.002 | 0.013 | 0.013 |
| " in volatile acids ... | " | 0.020 | 0.059 | 0.012 | 0.044 | 0.042 | 0.016 | 0.013 | 0.003 |
| " in non-volatile acids ... | " | 0.125 | 0.059 | 0.125 | 0.088 | 0.078 | 0.217 | 0.151 | 0.159 |
| " in volatile neutral compounds ... | " | 0.018 | 0.019 | 0.008 | 0.017 | 0.026 | 0.004 | 0.018 | 0.008 |
| " in synthetic compounds ... | " | 0.062 | 0.082 | 0.132 | 0.042 | 0.063 | 0.089 | 0.079 | 0.061 |
| Total carbon accounted for ... | " | 2.290 | 2.199 | 2.549 | 3.981 | 3.121 | 3.097 | 3.378 | 3.236 |
| " " in solution ... | " | 2.633 | 2.675 | 2.921 | 3.949 | 3.460 | 3.474 | 3.551 | 3.510 |
| Carbon unaccounted for (by difference) ... | " | 0.343 | 0.476 | 0.372 | Surplus of 0.032 | 0.339 | 0.377 | 0.173 | 0.274 |
| <i>Residual Glucose.</i> | | | | | | | | | |
| Glucose (by polarimeter) ... | per cent. | 1.010 | 1.061 | 1.212 | 1.826 | 1.527 | 1.202 | 1.498 | 1.548 |
| " (SHAFFER-HARTMANN) ... | " | 1.015 | 0.974 | 1.128 | 1.889 | 1.442 | 1.385 | 1.552 | 1.496 |
| " (WOOD-OST) ... | " | — | — | — | — | 1.473 | — | — | 1.498 |
| " (by alkaline iodine) ... | " | — | — | — | — | 1.470 | — | — | 1.549 |
| <i>Acids.</i> | | | | | | | | | |
| Titration (N/1 acid) ... | e.c. | Decrease of 0.7 | 0.2 | Decrease of 0.2 | Decrease of 0.2 | 0.1 | 0.1 | 0.3 | 0.1 |
| Volatile acids (N/1 acid) ... | " | 1.16 | 2.68 | 0.48 | 1.34 | 1.75 | — | — | 0.38 |
| Barium salts (weight) ... | gm. | — | 0.282 | 0.072 | 0.062 | 0.224 | — | 0.121 | 0.039 |
| Calcium salts (weight) ... | " | 0.966 | 0.922 | 1.198 | 0.470 | 0.374 | 0.790 | 0.677 | 0.660 |
| Volume of oxygen absorbed | " | 1655 | 1791 | 1523 | 928 | 1309 | 1423 | 1285 | 1400 |
| Respiration coefficient ... | " | 1.42 | 1.40 | 1.33 | 1.14 | 1.31 | 1.19 | 1.30 | 1.19 |
| Mycelium (weight) ... | gm. | 2.121 | 1.846 | 1.872 | 0.938 | 1.138 | 1.111 | 1.158 | 0.800 |
| " (carbon) ... | per cent. | 54.5 | 51.2 | 53.5 | 53.0 | 51.0 | 53.8 | 47.5 | 49.6 |

- (3) *A. versicolor*, Catalogue No. Ac. 28. Isolated at Ardeer from spoiled fuse and identified by Miss CHURCH.
- (4) *A. versicolor*, Catalogue No. Ac. 33. Purchased from Baarn.
- (5) *A. versicolor*, Catalogue No. Ac. 47.
- (6) *A. versicolor*, Catalogue No. Ac. 25.
- (7) *A. versicolor*, Catalogue No. Ac. 30.
- (8) *A. versicolor*, Catalogue No. Ac. 18.

Cultures Ac. 47, 25, 30 and 18 have all been purchased from different sources and supplied under other names (see p. 54). The carbon balance sheets for the eight strains of *A. versicolor* are given in Table VI.

This group does not show any striking biochemical characteristics, but it is apparent that all the balance sheets are of a similar nature. This may appear somewhat surprising when it is remembered that the eight different strains show great differences in cultural appearances, this being apparently characteristic of the *A. versicolor* group. All the cultures grow well on a synthetic medium and none of them produces any appreciable amount of end-products other than CO₂. Compared with other species of *Aspergillus* they do, however, give rise to medium amounts of volatile acids. None produces any appreciable amount of titratable acidity in the medium, and in some cases there is actually a decrease of the titration value compared with the unsown metabolism solution.

The following cultures of *A. Sydowi* BAINIER and SARTORY, were examined :—

- (1) *A. Sydowi*, Catalogue No. Ac. 89. Obtained from Dr. THOM and Miss CHURCH and bore their Catalogue No. Kop (KOPELOFF's) 22.
- (2) *A. Sydowi*, Catalogue No. Ac. 94. Purchased from the British National Collection of Type Cultures⁷⁵⁰ and bore the number Washington 3521.
- (3) *A. Sydowi*, Catalogue No. Ac. 99. Obtained from Mr. F. T. BROOKS of Cambridge and identified by Miss CHURCH.
- (4) *A. Sydowi*, Catalogue No. Ac. 59. Purchased from Baarn.
- (5) *A. Sydowi*, Catalogue No. Ac. 29. Isolated at Ardeer and identified by Miss CHURCH.
- (6) *A. cyaneus* BAINIER, Catalogue No. Ac. 97. Purchased from Baarn and in our opinion a strain of *A. Sydowi*.

The carbon balance sheets for these six species are given in Table VII.

All these balance sheets are of the same type as those of the *A. versicolor* strains and justify the placing of *A. Sydowi* in the same general group. All the cultures grow well on this medium and none of them gives rise to appreciable amounts of any end-product other than CO₂. They also resemble the *A. versicolor* species in the fact that none of them produces any appreciable amount of titratable acid, and that most of them actually give rise to a decrease in the titratable acidity.

TABLE VII.—Carbon balance sheets for *A. Sydowi* group.

| Species of <i>Aspergillus</i> : | <i>A. Sydowi</i> BAINIER and SARTORY. | | | | | <i>A. cyaneus</i> BAINIER. |
|---|---------------------------------------|----------------|----------------|--------|----------------|-------------------------------|
| Catalogue number : | Ac. 89 | Ac. 94 | Ac. 99 | Ac. 59 | Ac. 29 | Ac. 97 |
| Experiment number : | 124 | 127 | 131 | 89 | 38 | 129 |
| Incubation period in days : | 58 | 50 | 53 | 42 | 21 | 48 |
| <i>Carbon Balance Sheet.</i> | | | | | | |
| Carbon in solution (start) ... gm. | 5.043 | 5.043 | 5.043 | 4.834 | 5.094 | 5.043 |
| Carbon in H ₂ SO ₄ " | nil | 0.001 | nil | 0.001 | 0.001 | nil |
| " in CO ₂ " | 1.510 | 1.427 | 1.779 | 1.233 | 0.891 | 1.485 |
| " in mycelium " | 0.986 | 1.036 | 1.332 | 0.684 | 0.907 | 0.954 |
| " in solution (end) " | 2.345 | 2.406 | 1.751 | 2.826 | 3.265 | 2.438 |
| " accounted for " | 4.841 | 4.870 | 4.862 | 4.744 | 5.064 | 4.877 |
| " accounted for ... per cent. | 96.0 | 96.6 | 96.4 | 98.1 | 99.4 | 96.7 |
| <i>Analysis of Solution.</i> | | | | | | |
| Carbon in residual glucose ... gm. | 1.883 | 1.990 | 1.340 | 2.160 | 2.770 | 1.930 |
| " in CO ₂ in solution " | 0.025 | 0.028 | 0.026 | 0.010 | 0.008 | 0.017 |
| " in volatile acids " | 0.040 | 0.021 | 0.014 | nil | 0.005 | 0.019 |
| " in non-volatile acids " | 0.157 | 0.131 | 0.132 | 0.178 | 0.130 | 0.163 |
| " in volatile neutral compounds .. " | nil | 0.006 | 0.001 | 0.007 | 0.150 | 0.004 |
| " in synthetic compounds .. " | 0.126 | 0.066 | 0.124 | 0.071 | 0.078 | 0.172 |
| Total carbon accounted for " | 2.231 | 2.242 | 1.637 | 2.426 | 3.141 | 2.305 |
| " .. in solution " | 2.345 | 2.406 | 1.751 | 2.826 | 3.265 | 2.438 |
| Carbon unaccounted for (by difference) .. | 0.114 | 0.164 | 0.114 | 0.400 | 0.124 | 0.133 |
| <i>Residual Glucose.</i> | | | | | | |
| Glucose (by polarimeter) per cent. | 0.992 | 1.000 | 0.713 | 1.075 | 1.435 | 0.963 |
| " (SHAFFER-HARTMANN) .. " | 0.941 | 0.995 | 0.670 | 1.080 | 1.385 | 0.965 |
| " (WOOD-OST) " | 0.972 | 1.014 | 0.676 | 1.120 | — | 0.996 |
| " (by alkaline iodine) .. " | 1.046 | 1.022 | 0.728 | 1.115 | — | 0.994 |
| <i>Acids.</i> | | | | | | |
| | Decrease of | Decrease of | Decrease of | | Decrease of | |
| Titration (N/1 acid) c.c. | 0.1 | 0.4 | 0.9 | 0.9 | 0.9 | nil |
| Volatile acids (N/1 acid) " | 1.85 | 1.19 | 1.01 | 0.20 | 0.47 | 1.26 |
| Barium salts (weight) gm. | 0.176 | 0.073 | 0.053 | nil | 0.084 | 0.072 |
| Calcium salts (weight) " | 0.699 | 0.792 | 0.619 | 0.551 | 0.573 | 0.708 |
| Volume of oxygen absorbed c.c. | 2250 | 2135 | 2653 | 2068 | 1251 | 2297 |
| Respiration coefficient " | 1.27 | 1.27 | 1.27 | 1.12 | 1.34 | 1.22 |
| Mycelium (weight) gm. | 1.774 | 1.936 | 2.480 | 1.310 | 1.704 | 1.807 |
| " (carbon) per cent. | 55.6 | 53.5 | 53.7 | 52.2 | 56.6 | 52.8 |

Group 7.—*A. terreus*, *A. ustus* and connecting forms.

The following three species were included in this group :—

- (1) *A. terreus* THOM, Catalogue No. Ac. 24. Purchased from the British National Collection of Type Cultures (Catalogue No. 981) and bore the Washington Catalogue No. 144.
- (2) *A. terreus*, Catalogue No. Ac. 100. Obtained from Mr. F. T. BROOKS of Cambridge and identified by Miss CHURCH.
- (3) *A. ustus* BAINIER, Catalogue No. Ac. 84. Purchased from Baarn.

The carbon balance sheets of these three species are given in Table VIII.

It is evident from the carbon balance sheets that *A. terreus* and *A. ustus* are biochemically unrelated. The two different strains of *A. terreus* give similar balance sheets having the following characteristics: Both give rise to considerable amounts of volatile neutral compounds and have relatively high respiration coefficients. Neither of them gives rise to appreciable amounts of titratable acidity nor of volatile or non-volatile acids. On the other hand, *A. ustus* forms no volatile neutral compounds and has a respiration coefficient in the region of unity, whereas it produces a considerable amount of titratable acidity and a correspondingly large amount of carbon as non-volatile acids. Biochemically it is more closely related to the *A. Wentii* group than to *A. terreus*.

Group 8.—The white-spored *Aspergilli*, *A. flavipes*, *A. candidus* and connecting species toward the black *Aspergilli*.

Thanks to the courtesy of Dr. THOM and Miss CHURCH a considerable number of white species have been collected and examined. The species examined were as follows :—

- (1) *A. flavipes* BAINIER, Catalogue No. Ac. 73. Purchased from the British National Collection of Type Cultures and bore their Catalogue No. 1716.
- (2) *A. flavipes*, Catalogue No. Ac. 54. THOM and CHURCH Catalogue No. 4640·402.
- (3) *A. candidus* LINK, Catalogue No. Ac. 27. Purchased from the British National Collection of Type Cultures, Catalogue No. 595, and bore Catalogue No. Washington 106. This is apparently identical with (4).
- (4) *A. candidus*, Catalogue No. Ac. 48. THOM and CHURCH No. 106.
- (5) *A. albus* WILHELM, Catalogue No. Ac. 43. Purchased from Baarn.
- (6) *A. Okazakii* OKAZAKI, Catalogue No. Ac. 52. THOM and CHURCH Catalogue No. 4337, and referred to in their book, p. 158.
- (7) *A. species* (white), Catalogue No. Ac. 56. THOM and CHURCH Catalogue No. 4640·490.

TABLE VIII.—Carbon balance sheets for *A. terreus* and *A. ustus*.

| Species of <i>Aspergillus</i> : | | | | <i>A. terreus</i> THOM. | | <i>A. ustus</i> BAINIER. |
|---|--|--|--|-------------------------|---------|--------------------------|
| Catalogue number : | | | | Ac. 24 | Ac. 100 | Ac. 84 |
| Experiment number : | | | | 35 | 132 | 119 |
| Incubation period in days : | | | | 18 | 38 | 74 |
| <i>Carbon Balance Sheet.</i> | | | | | | |
| Carbon in solution (start) gm. | | | | 5.094 | 5.043 | 4.901 |
| Carbon in H ₂ SO ₄ " | | | | 0.001 | 0.024 | nil |
| " in CO ₂ " | | | | 0.999 | 1.636 | 1.919 |
| " in mycelium " | | | | 0.921 | 1.384 | 1.064 |
| " in solution (end) " | | | | 3.229 | 1.834 | 1.885 |
| " accounted for " | | | | 5.150 | 4.878 | 4.868 |
| " accounted for per cent. | | | | 101.1 | 96.7 | 99.3 |
| <i>Analysis of Solution.</i> | | | | | | |
| Carbon in residual glucose gm. | | | | 2.420 | 0.725 | 0.669 |
| " in CO ₂ in solution " | | | | 0.028 | 0.012 | 0.003 |
| " in volatile acids " | | | | 0.024 | 0.005 | — |
| " in non-volatile acids " | | | | 0.133 | 0.153 | 0.527 |
| " in volatile neutral compounds " | | | | 0.242 | 0.417 | 0.003 |
| " in synthetic compounds " | | | | 0.107 | 0.192 | 0.173 |
| Total carbon accounted for " | | | | 2.954 | 1.504 | 1.375 |
| " " in solution " | | | | 3.229 | 1.834 | 1.885 |
| Carbon unaccounted for (by difference) " | | | | 0.275 | 0.330 | 0.510 |
| <i>Residual Glucose.</i> | | | | | | |
| Glucose (by polarimeter) per cent. | | | | 1.267 | 0.290 | 0.412 |
| " (SHAFFER-HARTMANN) " | | | | 1.210 | 0.362 | 0.335 |
| " (WOOD-OST) " | | | | — | 0.363 | — |
| " (by alkaline iodine) " | | | | — | 0.416 | 0.381 |
| <i>Acids.</i> | | | | | | |
| Titration (N/1 acid) c.c. | | | | 0.8 | 0.0 | 8.8 |
| Volatile acids (N/1 acid) " | | | | 1.08 | 0.35 | — |
| Barium salts (weight) gm. | | | | 0.119 | 0.027 | — |
| Calcium salts (weight) " | | | | 0.799 | 0.580 | 1.797 |
| Volume of oxygen absorbed c.c. | | | | 1300 | 1759 | 3207 |
| Respiration coefficient " | | | | 1.48 | 1.75 | 1.12 |
| Mycelium (weight) gm. | | | | 1.642 | 2.453 | 2.059 |
| " (carbon) per cent. | | | | 56.1 | 56.4 | 51.7 |

- (8) *A. species* (white), Catalogue No. Ac. 55. THOM and CHURCH Catalogue No. 4640.489.
- (9) *A. species* (white), Catalogue No. Ac. 10. Isolated at Ardeer from a bench contamination of CZAPEK-DOX agar.
- (10) *A. species* (white), Catalogue No. Ac. 49. THOM and CHURCH Catalogue No. 117.
- (11) *A. species* (white), Catalogue No. Ac. 50. THOM and CHURCH Catalogue No. 4126.1.
- (12) *A. species* (white), Catalogue No. Ac. 51. THOM and CHURCH Catalogue No. 4261.
- (13) *A. species* (white), Catalogue No. Ac. 53. THOM and CHURCH Catalogue No. 4415.
- (14) *A. species* (white), Catalogue No. Ac. 101. Isolated at Ardeer and culturally apparently different from any of the other species.

Cultures Ac. 54, Ac. 48, Ac. 52, Ac. 56, Ac. 55, Ac. 49, Ac. 50, Ac. 51 and Ac. 53 were kindly given us by Dr. THOM and Miss CHURCH.

The carbon balance sheets for all these white species are given in Table IX.

The carbon balance sheets of the white species are biochemically very interesting, and entirely different in type from those of the black species. They show the following characteristics :—

(1) All the species are, in varying degree, difficult to grow on the synthetic medium used. Hence the experiments were of long duration and the weights of mycelium obtained were small in all cases.

(2) All species, with the possible exception of Ac. 101, give rise to only negligible amounts of titratable acidity, and in some cases to an actual decrease in the acidity. Some of the species, particularly Ac. 55 and Ac. 56, give rise to amounts of volatile acids which, while small in themselves, are large compared with those obtained from any other species of *Aspergillus* examined.

(3) None of the species produces appreciable amounts of non-volatile acids. This feature is in very marked contrast to the outstanding biochemical feature of the black *Aspergilli*.

(4) Almost all species give rise to a certain amount of volatile neutral compounds, though in no case is this very large. Thus the highest respiration coefficient is given by Ac. 27, *i.e.*, 1.58, while the average is about 1.3.

(5) All species give rise to considerable amounts of "carbon unaccounted for," and in three species, Ac. 56, Ac. 55 and Ac. 10, this is very considerable, amounting to about 45 per cent. of the glucose fermented in the case of Ac. 56. The "carbon unaccounted for" in these three cases has been investigated and has been shown to be largely mannitol (see Part IX).

In THOM and CHURCH's book *A. cinnamomeus* SCHIEMANN is included in this group (see p. 164). For reasons which are obvious on examination of its carbon balance sheet we have transferred it to the group of the black *Aspergilli*, *i.e.*, Group 9.

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TABLE IX.—Carbon balance sheets for white-spored *Aspergilli*.

| Species of <i>Aspergillus</i> : | <i>A. flavipes</i> BAINIER. | | <i>A. candidus</i> LINK. | | <i>A. albus</i> WILHELM. | <i>A.</i> <i>Okazaki</i> OKAZAKI. | <i>A. species</i> (white). | | | | | | | |
|--|-----------------------------|--------|--------------------------|-----------------------|-----------------------------|---|----------------------------|--------|--------|-----------------------|-----------------------|-----------------------|--------|---------|
| Catalogue number : | Ac. 73 | Ac. 54 | Ac. 27 | Ac. 48 | Ac. 43 | Ac. 52 | Ac. 56 | Ac. 55 | Ac. 10 | Ac. 49 | Ac. 50 | Ac. 51 | Ac. 53 | Ac. 101 |
| Experiment number : | 108 | 84 | 41 | 69 | 55 | 79 | 96 | 92 | 139 | 73 | 82 | 86 | 88 | 133 |
| Incubation period in days : | 47 | 34 | 36 | 65 | 36 | 31 | 48 | 46 | 51 | 40 | 35 | 40 | 46 | 90 |
| <i>Carbon Balance Sheet.</i> | | | | | | | | | | | | | | |
| Carbon in solution (start) ... gm. | 5.001 | 4.834 | 5.094 | 5.053 | 5.094 | 5.053 | 4.834 | 4.834 | 5.043 | 5.053 | 4.834 | 4.834 | 4.834 | 5.043 |
| Carbon in H ₂ SO ₄ ... | 0.003 | 0.002 | 0.005 | 0.019 | 0.001 | 0.001 | 0.002 | 0.001 | 0.002 | 0.002 | 0.001 | 0.001 | nil | 0.006 |
| " in CO ₂ ... | 1.169 | 0.819 | 1.407 | 1.136 | 0.501 | 1.109 | 1.209 | 1.513 | 1.734 | 0.803 | 1.265 | 0.941 | 0.841 | 1.133 |
| " in mycelium ... | 0.501 | 0.447 | 0.894 | 0.348 | 0.250 | 0.846 | 0.464 | 0.620 | 0.756 | 0.464 | 0.781 | 0.758 | 0.625 | 0.485 |
| " in solution (end) ... | 3.175 | 3.502 | 2.603 | 3.423 | 4.239 | 2.854 | 3.061 | 2.609 | 2.380 | 3.672 | 2.748 | 3.062 | 3.346 | 3.179 |
| " accounted for ... | 4.848 | 4.770 | 4.909 | 4.926 | 4.991 | 4.810 | 4.736 | 4.743 | 4.872 | 4.941 | 4.795 | 4.762 | 4.812 | 4.803 |
| " accounted for ... per cent. | 96.9 | 98.7 | 96.4 | 97.4 | 98.0 | 95.2 | 98.0 | 98.1 | 96.6 | 97.8 | 99.1 | 98.5 | 99.5 | 95.2 |
| <i>Analysis of Solution.</i> | | | | | | | | | | | | | | |
| Carbon in residual glucose ... gm. | 2.352 | 3.018 | 1.282 | 2.472 | 3.836 | 2.318 | 0.760 | 0.309 | 0.802 | 3.086 | 1.733 | 2.318 | 2.618 | 2.156 |
| " in CO ₂ in solution ... | 0.008 | 0.009 | 0.010 | 0.017 | 0.023 | 0.011 | 0.017 | 0.014 | 0.015 | 0.019 | 0.004 | 0.007 | 0.015 | 0.008 |
| " in volatile acids ... | 0.007 | 0.007 | 0.038 | 0.041 | 0.011 | nil | 0.071 | 0.094 | 0.022 | 0.010 | 0.006 | 0.001 | nil | 0.001 |
| " in non-volatile acids ... | 0.087 | 0.110 | 0.089 | 0.128 | 0.087 | 0.142 | 0.069 | 0.070 | 0.141 | 0.098 | 0.124 | 0.153 | 0.139 | 0.113 |
| " in volatile neutral compounds ... | 0.169 | 0.074 | 0.603 | 0.020 | 0.031 | 0.013 | 0.130 | 0.299 | 0.012 | 0.126 | 0.016 | 0.004 | 0.027 | 0.202 |
| " in synthetic compounds ... | 0.047 | 0.054 | 0.053 | 0.073 | 0.016 | 0.071 | 0.048 | 0.052 | 0.207 | 0.079 | 0.096 | 0.074 | 0.087 | 0.108 |
| Total carbon accounted for ... | 2.670 | 3.272 | 2.075 | 2.751 | 4.004 | 2.555 | 1.095 | 0.838 | 1.199 | 3.418 | 1.979 | 2.557 | 2.886 | 2.588 |
| " in solution ... | 3.175 | 3.502 | 2.603 | 3.423 | 4.239 | 2.854 | 3.061 | 2.609 | 2.380 | 3.672 | 2.748 | 3.062 | 3.346 | 3.179 |
| Carbon unaccounted for (by difference) ... | 0.505 | 0.230 | 0.528 | 0.672 | 0.235 | 0.299 | 1.966 | 1.771 | 1.181 | 0.254 | 0.769 | 0.505 | 0.460 | 0.591 |
| <i>Residual Glucose.</i> | | | | | | | | | | | | | | |
| Glucose (by polarimeter) ... per cent. | 1.227 | 1.465 | 0.620 | 1.218 | 2.055 | 1.156 | 0.534 | 0.125 | 0.445 | 1.602 | 0.912 | 1.155 | 1.213 | 1.044 |
| " (SHAFFER-HARTMANN) ... | 1.176 | 1.509 | 0.641 | 1.236 | 1.918 | 1.159 | 0.380 | 0.155 | 0.401 | 1.543 | 0.867 | 1.159 | 1.091 | 1.078 |
| " (WOOD-OST) ... | 1.240 | 1.510 | — | 1.246 | 1.896 | 1.174 | 0.432 | — | 0.390 | 1.490 | 0.918 | — | 1.140 | 1.138 |
| " (by alkaline iodine) ... | 1.288 | 1.550 | — | 1.330 | — | 1.196 | 0.436 | 0.177 | 0.464 | — | 0.926 | 1.216 | 1.126 | 1.174 |
| <i>Acids.</i> | | | | | | | | | | | | | | |
| Titration (N/1 acid) ... c.c. | 0.1 | 0.2 | nil | Decrease of 0.5 | Decrease of 0.2 | 0.8 | 0.8 | 0.5 | 0.2 | Decrease of 0.7 | Decrease of 0.5 | Decrease of 0.7 | 0.6 | 3.4 |
| Volatile acids (N/1 acid) ... | 0.39 | 1.16 | 1.34 | 1.74 | 1.17 | nil | 2.75 | 2.30 | 1.78 | 1.28 | 0.27 | 0.38 | nil | nil |
| Barium salts (weight) ... gm. | 0.073 | 0.025 | 0.186 | 0.155 | 0.035 | 0.006 | 0.350 | 0.313 | 0.062 | 0.047 | 0.014 | 0.008 | 0.002 | 0.029 |
| Calcium salts (weight) ... | 0.376 | 0.556 | 0.438 | 0.538 | 0.575 | 0.406 | 0.484 | 0.123 | 0.631 | 0.380 | 0.384 | 0.499 | 0.499 | 0.378 |
| Volume of oxygen absorbed ... c.c. | 1824 | 1500 | 1678 | 1944 | 845 | 1743 | 1617 | 2005 | 2611 | 1313 | 2091 | 1350 | 1245 | 1776 |
| Respiration coefficient ... | 1.20 | 1.03 | 1.58 | 1.11 | 1.16 | 1.20 | 1.41 | 1.42 | 1.25 | 1.17 | 1.13 | 1.31 | 1.28 | 1.20 |
| Mycelium (weight) ... gm. | 1.010 | 0.847 | 1.674 | 0.691 | 0.470 | 1.611 | 0.909 | 1.187 | 1.441 | 0.951 | 1.609 | 1.471 | 1.258 | 0.923 |
| " (carbon) ... per cent. | 49.6 | 52.8 | 53.4 | 50.4 | 53.2 | 52.5 | 51.1 | 52.2 | 52.5 | 48.8 | 48.5 | 51.6 | 49.7 | 52.5 |

(Facing p. 42.)

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TABLE X.—Carbon balance sheets for black *Aspergilli*—*A. niger* and allies.

| Species of <i>Aspergillus</i> : | <i>A. cinna- momeus</i> SCHIE- MANN. | <i>A. fuscus</i> SCHIE- MANN. | <i>A. luchuensis</i> T. INUI. | | | <i>A. violaceo- fuscus</i> GASP. | <i>A. niger</i> VAN TIEGHEM. | | | | <i>A. ficum</i> HENNING. | <i>A. niger citricus</i> WEHMER. | <i>A. pulver- ulentus</i> Mc- ALPINE. | <i>A. car- bonarius</i> BAINIER. | <i>A. fuma- ricus</i> WEHMER |
|---|--|-------------------------------------|-------------------------------|--------|--------|---|------------------------------|-------|-------|--------|-----------------------------|---|---|---|-------------------------------------|
| Catalogue number : | Ac. 42 | Ac. 37 | Ac. 66 | Ac. 88 | Ac. 96 | Ac. 44 | Ac. 1 | Ac. 2 | Ac. 4 | Ac. 13 | Ac. 3 | Ac. 72 | Ac. 12 | Ac. 11 | Ac. 103 |
| Experiment number : | 54 | 46 | 93 | 123 | 128 | 70 | 74 | 75 | 76 | 81 | 107 | 104 | 140 | 138 | 142 |
| Incubation period in days : | 13 | 44 | 44 | 49 | 41 | 25 | 14 | 24 | 22 | 23 | 29 | 22 | 39 | 26 | 58 |
| <i>Carbon Balance Sheet.</i> | | | | | | | | | | | | | | | |
| Carbon in solution (start) ... gm. | 5.094 | 5.094 | 4.834 | 5.043 | 5.043 | 5.053 | 5.053 | 5.053 | 5.053 | 4.834 | 5.001 | 4.834 | 5.043 | 5.043 | 4.944 |
| Carbon in H ₂ SO ₄ | 0.001 | 0.007 | 0.015 | 0.021 | 0.020 | 0.005 | 0.004 | 0.007 | 0.008 | 0.005 | 0.007 | 0.003 | 0.017 | 0.010 | nil |
| „ in CO ₂ | 1.029 | 1.295 | 1.841 | 2.011 | 1.940 | 1.041 | 0.714 | 1.182 | 1.066 | 1.095 | 1.125 | 0.834 | 1.785 | 1.229 | 1.755 |
| „ in mycelium | 0.768 | 0.363 | 0.516 | 0.542 | 0.556 | 0.534 | 0.369 | 0.614 | 0.444 | 0.671 | 0.260 | 0.352 | 0.786 | 0.308 | 0.764 |
| „ in solution (end) | 3.170 | 3.244 | 2.403 | 2.241 | 2.320 | 3.343 | 3.863 | 3.111 | 3.468 | 3.048 | 3.478 | 3.597 | 2.248 | 3.303 | 2.292 |
| „ accounted for | 4.968 | 4.909 | 4.775 | 4.815 | 4.836 | 4.923 | 4.950 | 4.914 | 4.986 | 4.819 | 4.870 | 4.786 | 4.836 | 4.850 | 4.811 |
| „ accounted for ... per cent. | 97.5 | 96.4 | 98.8 | 95.5 | 95.9 | 97.4 | 97.9 | 97.2 | 98.6 | 99.7 | 97.4 | 99.0 | 95.8 | 95.8 | 97.3 |
| <i>Analysis of Solution.</i> | | | | | | | | | | | | | | | |
| Carbon in residual glucose ... gm. | 2.332 | 1.103 | 0.419 | 0.042 | 0.266 | 1.859 | 2.890 | 1.650 | 1.599 | 1.966 | 1.902 | 2.454 | 0.098 | 1.734 | 1.042 |
| „ in CO ₂ in solution | 0.007 | 0.004 | 0.006 | 0.007 | 0.005 | 0.003 | 0.009 | 0.005 | 0.003 | 0.006 | 0.010 | 0.008 | 0.001 | 0.003 | nil |
| „ in volatile acids | nil | 0.016 | nil | 0.006 | 0.004 | 0.047 | 0.006 | 0.006 | 0.003 | 0.006 | 0.001 | nil | nil | 0.009 | nil |
| „ in non-volatile acids | 0.453 | 0.364 | 0.247 | 0.177 | 0.169 | 0.260 | 0.163 | 0.210 | 0.531 | 0.233 | 0.099 | 0.300 | 0.654 | 0.166 | 0.590 |
| „ in volatile neutral compounds .. | 0.069 | 1.077 | 1.118 | 1.541 | 1.342 | 0.618 | 0.620 | 0.742 | 0.857 | 0.396 | 1.072 | 0.475 | 1.032 | 0.907 | 0.012 |
| „ in synthetic compounds .. | 0.148 | 0.071 | 0.084 | 0.166 | 0.131 | 0.118 | 0.070 | 0.102 | 0.131 | 0.098 | 0.072 | 0.072 | 0.295 | 0.065 | 0.243 |
| Total carbon accounted for | 3.009 | 2.635 | 1.874 | 1.939 | 1.917 | 2.905 | 3.758 | 2.715 | 3.124 | 2.705 | 3.156 | 3.309 | 2.080 | 2.884 | 1.887 |
| „ „ in solution | 3.170 | 3.244 | 2.403 | 2.241 | 2.320 | 3.343 | 3.863 | 3.111 | 3.468 | 3.048 | 3.478 | 3.597 | 2.248 | 3.303 | 2.292 |
| Carbon unaccounted for (by difference) .. | 0.161 | 0.609 | 0.529 | 0.302 | 0.403 | 0.438 | 0.105 | 0.396 | 0.344 | 0.343 | 0.322 | 0.288 | 0.163 | 0.419 | 0.405 |
| <i>Residual Glucose.</i> | | | | | | | | | | | | | | | |
| Glucose (by polarimeter) ... per cent. | 1.302 | 0.582 | 0.219 | 0.018 | 0.122 | 0.952 | 1.357 | 0.854 | 0.819 | 1.006 | 1.021 | 1.227 | 0.078 | 0.920 | 0.608 |
| „ (SHAFFER-HARTMANN) .. | 1.166 | 0.551 | 0.210 | 0.021 | 0.133 | 0.930 | 1.445 | 0.825 | 0.800 | 0.983 | 0.951 | 1.227 | 0.049 | 0.867 | 0.521 |
| „ (WOOD-OST) | — | — | — | — | — | 0.903 | 1.410 | 0.822 | 0.790 | 1.014 | 0.970 | 1.210 | — | 0.908 | 0.561 |
| „ (by alkaline iodine) .. | — | — | 0.315 | 0.114 | 0.325 | — | — | — | — | 1.050 | 0.961 | 1.245 | 0.102 | 0.953 | 0.493 |
| <i>Acids.</i> | | | | | | | | | | | | | | | |
| Titration (N/1 acid) c.c. | 29.3 | 7.5 | 5.7 | 2.9 | 3.6 | 8.8 | 5.1 | 5.2 | 21.3 | 8.3 | 3.1 | 10.2 | 20.8 | 5.0 | 16.8 |
| Volatile acids (N/1 acid) | — | 4.32 | nil | 0.63 | 0.48 | 1.91 | nil | 1.04 | 0.47 | 0.41 | 0.14 | nil | nil | nil | 0.57 |
| Barium salts (weight) gm. | 0.009 | 0.110 | 0.021 | 0.032 | 0.017 | 0.231 | 0.027 | 0.010 | 0.008 | 0.047 | 0.021 | nil | 0.018 | 0.021 | 0.028 |
| Calcium salts (weight) | 1.720 | 1.436 | 0.939 | 0.744 | — | 1.079 | 0.774 | 1.000 | 2.536 | 1.169 | 0.751 | 1.308 | 2.934 | 0.898 | 1.913 |
| Volume of oxygen absorbed ... c.c. | 1944 | 1375 | 2267 | 2019 | 2074 | 1191 | 697 | 1265 | 1270 | 1660 | 1081 | 1080 | 2131 | 1367 | 3116 |
| Respiration coefficient | 0.99 | 1.76 | 1.52 | 1.87 | 1.75 | 1.64 | 1.93 | 1.75 | 1.57 | 1.24 | 1.96 | 1.45 | 1.57 | 1.68 | 1.05 |
| Mycelium (weight) gm. | 1.608 | 0.736 | 1.079 | 1.099 | 1.181 | 1.092 | 0.747 | 1.211 | 0.889 | 1.286 | 0.525 | 0.698 | 1.506 | 0.645 | 1.621 |
| „ (carbon) per cent. | 47.8 | 49.3 | 47.8 | 49.3 | 47.1 | 48.9 | 49.4 | 50.7 | 49.9 | 52.2 | 49.6 | 50.5 | 52.2 | 47.8 | 47.1 |

(Facing p. 43.)

Group 9.—The black *Aspergilli*, *A. niger* and allies.

A large number of species belonging to this group, which is a very well marked one biochemically, have been examined. They are :—

- (1) *A. cinnamomeus* SCHIEMANN, Catalogue No. Ac. 42. Purchased from Baarn.
- (2) *A. fuscus* SCHIEMANN, Catalogue No. Ac. 37. Purchased from Baarn.
- (3) *A. luchuensis* T. INUI, Catalogue No. Ac. 66. Purchased from Baarn.
- (4) *A. luchuensis*, Catalogue No. Ac. 88. Sent by Miss CHURCH and bore the Catalogue Number 4291.3 (see THOM and CHURCH's book, p. 171).
- (5) *A. luchuensis*, Catalogue No. Ac. 96. Purchased from the British National Collection of Type Cultures, Catalogue No. 1017, and bore the number Washington 1491.3. ? 4291.3
- (6) *A. violaceo-fuscus* GASPERINI, Catalogue No. Ac. 44. Purchased from Baarn as *A. atropurpureus*, was identified by Miss CHURCH as definitely not this species, and was given by her as a strain of *A. violaceo-fuscus* (THOM and CHURCH's book, pp. 172 and 180, where it bears the culture number 4724.44).
- (7) *A. niger* VAN TIEGHEM, Catalogue No. Ac. 1. Obtained from Dr. PAINE.
- (8) *A. niger*, Catalogue No. Ac. 2. Obtained from Dr. THOM and bore the number 111.
- (9) *A. niger*, Catalogue No. Ac. 4. Obtained from Dr. THOM and bore the number 3528.7.
- (10) *A. niger*, Catalogue No. Ac. 13. Purchased from the British National Collection of Type Cultures, No. 594.
- (11) *A. ficuum* HENNINGS, Catalogue No. Ac. 3. Received from Dr. THOM, No. 142.
- (12) *A. niger citricus* WEHMER, Catalogue No. Ac. 72. Purchased from the British National Collection of Type Cultures, No. 1692.
- (13) *A. pulverulentus* MCALPINE, Catalogue No. Ac. 12. Purchased from the British National Collection of Type Cultures, No. 1324.
- (14) *A. carbonarius* BAINIER, Catalogue No. Ac. 11. Purchased from the British National Collection of Type Cultures, No. 1325.
- (15) *A. fumaricus* WEHMER, Catalogue No. Ac. 103. Sent by Dr. THOM and Miss CHURCH and bore their number 4668.2 (see THOM and CHURCH's book, p. 181).

The carbon balance sheets for these 15 cultures are given in Table X.

The carbon balance sheets for the *A. niger* group show immediately the vast biochemical difference between this group and the white species of *Aspergillus*. The two groups differ in almost every particular.

The *A. niger* group shows the following biochemical characteristics :—

- (1) All species in this group grow readily on the synthetic medium used and metabolize the glucose supplied in a relatively short time.
- (2) In direct contrast to the white species, all species in this group give rise to large amounts of titratable acid and, with the possible exception of Ac. 44, none of them produces any volatile acids.

(3) This group is chiefly distinguished biochemically by the large amounts of non-volatile acids produced, all the titratable acidity being present apparently as non-volatile acid. The production of acids of this type by the *A. niger* group has, of course, been long known, *e.g.*, the production of oxalic, citric and gluconic acids by different strains of *A. niger* and of fumaric acid by *A. fumaricus*.

(4) Quantitatively, the largest yield of material produced by most of the species in this group is indicated under the heading "volatile neutral compounds" and has been shown to be alcohol. This is reflected in the high respiration coefficients of almost all the species. The average yield of alcohol is considerably higher than with the white species, and seems to put this group into line with the *A. flavus-oryzæ* group.

(5) While some of the species in this group give moderate amounts of "carbon unaccounted for," none of them gives a yield of this type of product comparable with that obtained from some of the white species.

(6) *A. cinnamomeus* and *A. fumaricus* have similar characteristics, and are both somewhat different from the average members of this group.

Group 10.—Yellow, ochraceous and brown *Aspergilli*.

THOM and CHURCH arrange the species in this group under three headings :—

- (a) *A. Wentii* group.
- (b) *A. ochraceus* group.
- (c) *A. tamaris* group.

As a result of the preparation of carbon balance sheets for a number of species in this group it is apparent that biochemically, at any rate, they form three distinct sub-groups, *A. Wentii* forming a separate group having very marked characteristics of its own, while *A. tamaris* seems to be more closely allied to the *A. flavus-oryzæ* group than to the *A. ochraceus* group. Hence these three sub-groups will be dealt with separately.

(a) *A. Wentii* group.—The following three strains have been examined :—

- (1) *A. Wentii* WEHMER, Catalogue No. Ac. 34. Purchased from Baarn.
- (2) *A. Wentii*, Catalogue No. Ac. 81. Sent by Miss CHURCH and labelled 4202·16 C.
- (3) *A. Wentii*, Catalogue No. Ac. 82. Sent by Miss CHURCH and labelled 4230.

The carbon balance sheets for these three strains are given in Table XI.

The type of carbon balance sheets given by these three strains, which agree closely amongst themselves, is very similar to that given by the *A. glaucus* group. *A. Wentii* differs from the members of the *A. glaucus* group in the ease with which it grows on this synthetic solution, but apart from this the resemblance is very marked. Thus, *A. Wentii* gives considerable amounts of titratable acids which consist entirely of non-volatile acids, the production of volatile acids by this group being apparently nil. *A. Wentii* also gives rise to some material (probably a non-volatile acid) which is optically dextro-rotatory and does not reduce copper solutions. *A. Wentii* further resembles

TABLE XI.—Carbon balance sheets for *A. Wentii* group.

| Species of <i>Aspergillus</i> : | | | | <i>A. Wentii</i> WEHMER. | | |
|---|--|--|--|--------------------------|--------|--------|
| Catalogue number : | | | | Ac. 34 | Ac. 81 | Ac. 82 |
| Experiment number : | | | | 43 | 114 | 115 |
| Incubation period in days : | | | | 27 | 51 | 53 |
| <i>Carbon Balance Sheet.</i> | | | | | | |
| Carbon in solution (start) gm. | | | | 5.094 | 5.001 | 5.001 |
| Carbon in H ₂ SO ₄ " | | | | nil | 0.002 | nil |
| " in CO ₂ " | | | | 1.064 | 1.207 | 1.187 |
| " in mycelium " | | | | 0.724 | 0.536 | 0.690 |
| " in solution (end) " | | | | 3.155 | 3.208 | 3.058 |
| " accounted for " | | | | 4.943 | 4.953 | 4.935 |
| " accounted for per cent. | | | | 97.0 | 99.1 | 98.7 |
| <i>Analysis of Solution.</i> | | | | | | |
| Carbon in residual glucose gm. | | | | 2.430 | 1.602 | 1.451 |
| " in CO ₂ in solution " | | | | 0.001 | 0.005 | 0.003 |
| " in volatile acids " | | | | 0.003 | nil | nil |
| " in non-volatile acids " | | | | 0.316 | 0.537 | 0.568 |
| " in volatile neutral compounds " | | | | 0.006 | 0.013 | 0.009 |
| " in synthetic compounds " | | | | 0.094 | 0.140 | 0.117 |
| Total carbon accounted for " | | | | 2.850 | 2.297 | 2.148 |
| " " in solution " | | | | 3.155 | 3.208 | 3.058 |
| Carbon unaccounted for (by difference) " | | | | 0.305 | 0.911 | 0.910 |
| <i>Residual Glucose.</i> | | | | | | |
| Glucose (by polarimeter) per cent. | | | | 1.331 | 1.046 | 1.033 |
| " (SHAFFER-HARTMANN) " | | | | 1.215 | 0.801 | 0.725 |
| " (WOOD-OST) " | | | | — | 0.818 | 0.737 |
| " (by alkaline iodine) " | | | | — | 0.841 | 0.750 |
| <i>Acids.</i> | | | | | | |
| Titration (N/1 acid) c.c. | | | | 2.3 | 9.7 | 11.5 |
| Volatile acids (N/1 acid) " | | | | 1.49 | nil | nil |
| Barium salts (weight) gm. | | | | 0.025 | 0.035 | 0.012 |
| Calcium salts (weight) " | | | | 1.140 | 1.985 | 2.084 |
| Volume of oxygen absorbed c.c. | | | | 1670 | 2006 | 2151 |
| Respiration coefficient " | | | | 1.19 | 1.13 | 1.03 |
| Mycelium (weight) gm. | | | | 1.407 | 1.087 | 1.415 |
| " (carbon) per cent. | | | | 51.7 | 49.4 | 48.8 |

the *A. glaucus* group in not giving rise to volatile neutral compounds (alcohol), and hence in having a respiration coefficient approximating to unity. The production of large amounts of "carbon unaccounted for" by two out of the three strains of *A. Wentii* is also another feature which it has in common with the *A. glaucus* group. The nature of the metabolic products of *A. Wentii* is dealt with in Part XVII.

(b) *A. ochraceus* group.—Carbon balance sheets were prepared for the following species placed in this group :—

- (1) *A. ochraceus* WILHELM, Catalogue No. Ac. 23. Purchased from the British National Collection of Type Cultures, No. 979. Washington No. 4065.1.
- (2) *A. ochraceus*, Catalogue No. Ac. 63. Sent by Mr. F. T. BROOKS of Cambridge.
- (3) *A. helvus* BAINIER, Catalogue No. Ac. 83. Purchased from Baarn.
- (4) *A. elegans* GASPERINI, Catalogue No. Ac. 40. Purchased from Baarn.
- (5) *A. ostianus* WEHMER, Catalogue No. Ac. 35. Purchased from Baarn.
- (6) *A. terricola* var. *Americana* MARCHAL, Catalogue No. Ac. 22. Purchased from the British National Collection of Type Cultures, No. 974.

The carbon balance sheets for these six species are given in Table XII.

The general type of balance sheet in this group is so similar to that of the *A. flavus-oryzæ* group (Group 11) that observations on both the groups will be given in the *A. flavus-oryzæ* group. The *A. ochraceus* group is, however, distinguished biochemically from the *A. flavus-oryzæ* group by the fact that none of the species in our possession which are placed in the *A. ochraceus* group gives rise to kojic acid, and, as indicated in Part VII, we regard the production of kojic acid as being diagnostic for members of the *A. flavus-oryzæ* group.

Group 11.—The yellow-green *Aspergilli*, *A. flavus-oryzæ*.

The following species are included in this group :—

- (1) *A. tamarii* KITA, Catalogue No. Ac. 26. Purchased from the British National Collection of Type Cultures, No. 599. Washington 423512. 4235 x.l.; 4235.12
- (2) *A. tamarii*, Catalogue No. Ac. 62. Sent by Mr. F. T. BROOKS of Cambridge.
- (3) *A. oryzae* AHLBURG, Catalogue No. Ac. 19. Purchased from the British National Collection of Type Cultures, No. 598. Washington 113.
- (4) *A. parasiticus* SPEARE, Catalogue No. Ac. 14. Purchased from the British National Collection of Type Cultures, No. 975. Washington 3509.
- (5) *A. flavus* LINK, Catalogue No. Ac. 16. Purchased from the British National Collection of Type Cultures, No. 596. Washington 108.
- (6) *A. flavus*, Catalogue No. Ac. 91. Isolated at Ardeer from mouldy "Pegamoid" leather cloth.
- (7) *A. effusus* TIRABOSCHI, Catalogue No. Ac. 21. Purchased from the British National Collection of Type Cultures, No. 973. Washington 130.

Carbon balance sheets for these seven species are given in Table XIII.

TABLE XII.—Carbon balance sheets for *A. ochraceus* group.

| Species of <i>Aspergillus</i> : | <i>A. ochraceus</i> WILHELM. | | | <i>A. helvus</i> BAINIER. | | <i>A. elegans</i> GASPERINI. | | <i>A. ostianus</i> WEIMER. | | <i>A. terricola</i> var. <i>Americana</i> MARCHAL. | |
|--|------------------------------|--------|--------|---------------------------|--------|------------------------------|--------|----------------------------|--------|--|--------|
| | Ac. 23 | Ac. 23 | Ac. 63 | Ac. 83 | Ac. 40 | Ac. 40 | Ac. 35 | Ac. 35 | Ac. 22 | Ac. 22 | Ac. 22 |
| Catalogue number : | 34 | 78 | 87 | 117 | 52 | 68 | 48 | 67 | 27 | 85 | |
| Experiment number : | 19 | 30 | 32 | 44 | 25 | 38 | 31 | 34 | 13 | 30 | |
| Incubation period in days : | | | | | | | | | | | |
| <i>Carbon Balance Sheet.</i> | | | | | | | | | | | |
| Carbon in solution (start) ... gm. | 5.094 | 5.053 | 4.834 | 4.901 | 5.053 | 5.094 | 5.094 | 4.875 | 5.020 | 4.834 | |
| Carbon in H ₂ SO ₄ ... " | 0.001 | 0.005 | 0.002 | 0.017 | 0.009 | 0.006 | 0.005 | 0.007 | 0.010 | 0.013 | |
| " in CO ₂ ... " | 1.011 | 1.243 | 1.514 | 1.691 | 1.743 | 1.381 | 1.788 | 1.401 | 1.311 | 1.048 | |
| " in mycelium ... " | 0.818 | 0.688 | 1.396 | 0.857 | 0.618 | 0.636 | 1.083 | 0.608 | 0.445 | 0.255 | |
| " in solution (end) ... " | 3.173 | 2.994 | 1.826 | 2.246 | 2.591 | 2.969 | 1.958 | 2.859 | 3.187 | 3.403 | |
| " accounted for ... " | 5.003 | 4.930 | 4.738 | 4.811 | 4.961 | 4.992 | 4.834 | 4.875 | 4.953 | 4.719 | |
| " accounted for ... per cent. | 98.2 | 97.5 | 98.0 | 98.1 | 98.1 | 98.0 | 94.9 | 96.4 | 98.7 | 97.6 | |
| <i>Analysis of Solution.</i> | | | | | | | | | | | |
| Carbon in residual glucose ... gm. | 2.476 | 1.794 | 0.912 | 0.455 | 0.336 | 1.174 | 0.813 | 1.375 | 1.790 | 1.447 | |
| " in CO ₂ in solution ... " | 0.020 | 0.015 | 0.018 | 0.015 | 0.001 | 0.008 | 0.004 | nil | 0.007 | 0.009 | |
| " in volatile acids ... " | 0.036 | 0.024 | 0.055 | 0.028 | 0.047 | 0.046 | 0.006 | 0.018 | 0.010 | 0.037 | |
| " in non-volatile acids ... " | 0.098 | 0.136 | 0.113 | 0.101 | 0.135 | 0.145 | 0.113 | 0.123 | 0.119 | 0.141 | |
| " in volatile neutral compounds ... " | 0.117 | 0.507 | 0.194 | 1.012 | 0.758 | 0.710 | 0.461 | 0.670 | 0.907 | 1.424 | |
| " in synthetic compounds ... " | 0.081 | 0.053 | 0.050 | 0.111 | 0.066 | 0.067 | 0.042 | 0.058 | 0.114 | 0.083 | |
| Total carbon accounted for ... " | 2.828 | 2.529 | 1.342 | 1.722 | 1.343 | 2.150 | 1.439 | 2.244 | 2.947 | 3.141 | |
| " in solution ... " | 3.173 | 2.994 | 1.826 | 2.246 | 2.591 | 2.969 | 1.958 | 2.859 | 3.187 | 3.403 | |
| Carbon unaccounted for (by difference) ... " | 0.345 | 0.465 | 0.484 | 0.524 | 1.248 | 0.819 | 0.519 | 0.615 | 0.240 | 0.262 | |
| <i>Residual Glucose.</i> | | | | | | | | | | | |
| Glucose (by polarimeter) ... per cent. | 1.230 | 0.914 | 0.447 | 0.240 | 0.108 | 0.878 | — | 0.688 | 0.850 | 0.666 | |
| " (SHAFFER-HARTMANN) ... " | 1.238 | 0.897 | 0.456 | 0.228 | 0.168 | 0.587 | 0.407 | 0.688 | 0.845 | 0.724 | |
| " (WOOD-OST) ... " | — | 0.914 | 0.480 | — | — | — | — | 0.691 | — | 0.730 | |
| " (by alkaline iodine) ... " | — | — | 0.486 | 0.290 | — | — | — | — | — | 0.777 | |
| <i>Acids.</i> | | | | | | | | | | | |
| Titration (N/1 acid) ... c.c. | 0.5 | 1.4 | 0.1 | 1.2 | 2.0 | 0.3 | 1.0 | 1.4 | 4.2 | 5.1 | |
| Volatile acids (N/1 acid) ... " | 1.25 | 1.04 | 2.31 | 2.56 | 1.91 | 3.13 | 1.13 | 0.20 | — | 1.99 | |
| Barium salts (weight) ... gm. | 0.271 | 0.086 | 0.310 | 0.159 | 0.252 | 0.234 | 0.110 | 0.073 | — | 0.182 | |
| Calcium salts (weight) ... " | 0.448 | 0.624 | 0.523 | 0.629 | 0.631 | 0.701 | 0.440 | 0.618 | 0.858 | 0.446 | |
| Volume of oxygen absorbed ... c.c. | 1325 | 1557 | 1790 | 1639 | 2063 | 1438 | 2354 | 1582 | 1413 | 649 | |
| Respiration coefficient ... " | 1.45 | 1.53 | 1.60 | 1.94 | 1.58 | 1.81 | 1.42 | 1.65 | 1.74 | 3.04 | |
| Mycelium (weight) ... gm. | 1.589 | 1.379 | 2.414 | 1.590 | 1.236 | 1.348 | 2.054 | 1.119 | 0.850 | 0.488 | |
| " (carbon) ... per cent. | 52.5 | 49.9 | 57.8 | 53.9 | 50.0 | 47.2 | 52.7 | 54.3 | 52.4 | 52.2 | |

TABLE XIII.—Carbon balance sheets for yellow-green *Aspergilli*.

| Species of <i>Aspergillus</i> : | <i>A. tamarii</i> KITA. | | <i>A. oryzae</i> AHLBURG. | <i>A. parasiticus</i> SPEARE. | <i>A. flavus</i> LINK. | | <i>A. effusus</i> TIRA- BOSCHI. |
|--|-------------------------|--------|------------------------------|----------------------------------|------------------------|--------|---------------------------------------|
| Catalogue number : | Ac. 26 | Ac. 62 | Ac. 19 | Ac. 14 | Ac. 16 | Ac. 91 | Ac. 21 |
| Experiment number : | 36 | 83 | 18 | 19 | 31 | 125 | 25 |
| Incubation period in days : | 20 | 30 | 15 | 22 | 22 | 35 | 16 |
| <i>Carbon Balance Sheet.</i> | | | | | | | |
| Carbon in solution (start) ... gm. | 5.094 | 4.834 | 5.020 | 5.020 | 5.094 | 5.043 | 5.020 |
| Carbon in H ₂ SO ₄ ... | 0.001 | 0.004 | 0.017 | 0.003 | 0.014 | 0.010 | 0.062 |
| „ in CO ₂ ... | 0.992 | 1.062 | 1.576 | 1.373 | 1.121 | 1.435 | 1.390 |
| „ in mycelium ... | 0.469 | 0.445 | 0.256 | 0.762 | 0.225 | 0.595 | 0.410 |
| „ in solution (end) ... | 3.508 | 3.240 | 3.152 | 2.792 | 3.576 | 2.755 | 3.133 |
| „ accounted for ... | 4.970 | 4.751 | 5.001 | 4.930 | 4.936 | 4.795 | 4.995 |
| „ accounted for ... per cent. | 97.6 | 98.3 | 99.6 | 98.2 | 96.9 | 95.1 | 99.5 |
| <i>Analysis of Solution.</i> | | | | | | | |
| Carbon in residual glucose ... gm. | 2.798 | 2.268 | 1.206 | 1.580 | 1.714 | 1.179 | 1.878 |
| „ in CO ₂ in solution ... | 0.019 | 0.011 | 0.004 | 0.003 | 0.011 | 0.015 | 0.003 |
| „ in volatile acids ... | 0.022 | 0.007 | 0.050 | 0.008 | 0.014 | 0.012 | 0.026 |
| „ in non-volatile acids ... | 0.128 | 0.117 | 0.091 | 0.136 | 0.173 | 0.090 | 0.148 |
| „ in volatile neutral compounds ... | 0.288 | 0.489 | 1.348 | 0.305 | 1.483 | 1.144 | 0.517 |
| „ in synthetic compounds ... | 0.125 | 0.048 | 0.039 | 0.101 | 0.091 | 0.117 | 0.225 |
| Total carbon accounted for ... | 3.380 | 2.940 | 2.738 | 2.133 | 3.486 | 2.557 | 2.797 |
| „ „ in solution... .. | 3.508 | 3.240 | 3.152 | 2.792 | 3.576 | 2.755 | 3.133 |
| Carbon unaccounted for (by difference) .. | 0.128 | 0.300 | 0.414 | 0.659 | 0.090 | 0.198 | 0.336 |
| <i>Residual Glucose.</i> | | | | | | | |
| Glucose (by polarimeter) ... per cent. | 1.496 | 1.171 | 0.590 | 0.790 | 0.882 | 0.647 | 0.860 |
| „ (SHAFFER-HARTMANN) ... | 1.399 | 1.134 | 0.603 | 0.790 | 0.857 | 0.589 | 0.939 |
| „ (WOOD-OST) ... | — | 1.158 | — | — | — | 0.581 | — |
| „ (by alkaline iodine) ... | — | 1.168 | — | 2.290 | — | 0.669 | — |
| <i>Acids.</i> | | | | | | | |
| Titration (N/1 acid) ... c.c. | 2.0 | 1.5 | 2.7 | 0.9 | 6.9 | 1.3 | Decrease of 0.3 |
| Volatile acids (N/1 acid) ... | 1.51 | 1.46 | 2.31 | 0.13 | 0.53 | 1.08 | 1.48 |
| Barium salts (weight) ... gm. | 0.122 | 0.030 | — | — | 0.105 | 0.051 | 0.078 |
| Calcium salts (weight) ... | 0.572 | 0.565 | 0.611 | 0.422 | 0.623 | 0.517 | 0.596 |
| Volume of oxygen absorbed ... c.c. | 1339 | 1413 | 1441 | 1710 | 824 | 1228 | 1915 |
| Respiration coefficient ... | 1.41 | 1.42 | 2.05 | 1.50 | 2.56 | 2.21 | 1.36 |
| Mycelium (weight) ... gm. | 0.992 | 0.870 | 0.560 | 1.751 | 0.456 | 1.120 | 0.771 |
| „ (carbon) ... per cent. | 47.3 | 51.1 | 45.8 | 43.5 | 49.4 | 53.2 | 53.2 |

The majority of the members of both the *A. ochraceus* group and *A. flavus-oryzæ* group grow well on the synthetic medium used. They produce only moderate amounts of titratable acidity, and only small amounts of non-volatile acids. Generally speaking, the majority produce amounts of volatile acids which, while small in themselves, are well above the average for the majority of species of *Aspergillus*. The outstanding features of this group are, however, (a) the production of volatile neutral compounds (alcohol) and, in consequence, high respiration coefficients; and (b) the production of kojic acid.

(a) The production of alcohol by certain members of the *A. flavus-oryzæ* group is, of course, well known, since strains of *A. oryzæ* have long been used in Japan in the manufacture of alcoholic liquors. It is interesting, however, to find that this characteristic persists throughout both groups although, of course, it is less marked in some species than in others. The *A. ochraceus* and the *A. flavus-oryzæ* groups are closely allied biochemically to the *A. niger* group, but are sharply marked off from the last group by the fact that, whereas they produce relatively small amounts of titratable acid, the *A. niger* group gives rise to considerable quantities.

(b) The production of kojic acid by members of this group has been examined in detail and is reported in Part VII. It may be mentioned, however, that, of the species of this group in our possession, *A. parasiticus* SPEARE, Ac. 14, gives the best yields of kojic acid (16–20 per cent.). This is indicated in the balance sheets given in Table XIII, in which *A. parasiticus* has the highest figure for “carbon unaccounted for” ($0.659 \equiv 19.1$ per cent. of the glucose utilised) since kojic acid is included in the type of carbon compounds classed as “carbon unaccounted for.” Reference may also be made to the large difference between the figures for glucose by polarimeter (0.79 per cent.) and glucose by alkaline iodine (2.29 per cent.).

Discussion of results obtained.

A summary of all the carbon balance sheets obtained for species of *Aspergillus* is given in Tables XIV and XV.

In Table XIV the original eleven groups are further grouped into six sections, according to the types of products formed, and it is important to note that the figures given in this table are the *average* of figures arrived at from *all* the carbon balance sheets given in that particular group. Thus, by way of example, the figure for carbon in “volatile neutral compounds” (1.247) given for Group 1, *i.e.*, the *A. clavatus* group, is the average from the six carbon balance sheets given in Table I, *i.e.*, the average of 1.629, 2.064, 1.554, 0.835, 0.807, 0.594. In Table XV the sections are arranged as in Table XIV, but the values given in this table are the *limiting figures* given in the corresponding balance sheets. Thus the values given for the same example chosen above, *i.e.*, the volatile neutral compounds formed by Group 1—the *A. clavatus* group—are 2.064–0.594.

TABLE XIV.—Summary of carbon balance sheets for species of *Aspergillus*, arranged according to type of products.
Average values.

| No. of Group. | Name of Group. | Carbon in volatile neutral compounds. | Respiration coefficient. | Titration. N/1 acid. | Carbon in non-volatile acids. | Carbon in volatile acids. | Carbon unaccounted for. | Growth on Czapek-Dox metabolism solution. | Carbon in mycelium. | No. of experiments averaged. |
|---------------|--------------------------|---------------------------------------|--------------------------|----------------------|-------------------------------|---------------------------|-------------------------|---|---------------------|------------------------------|
| 1 | <i>Clavatus</i> | gm. 1.247 | 1.91 | c.c. 2.0 | gm. 0.092 | gm. 0.013 | gm. 0.386 | Good | Per cent. 51.0 | 6 |
| 11 | <i>Flavus-oryzae</i> | ... 0.796 | 1.79 | 2.1 | 0.126 | 0.020 | 0.304 | Good | 49.1 | 7 |
| 10 | <i>Ochraceus</i> | ... 0.676 | 1.78 | 1.7 | 0.122 | 0.031 | 0.552 | Good | 52.3 | 10 |
| 5 | <i>Nidulans</i> (Type A) | ... 0.843 | 1.63 | 1.3 | 0.082 | 0.024 | 0.745 | Good | 49.8 | 3 |
| 7 | <i>Terreus</i> | ... 0.330 | 1.62 | 0.4 | 0.143 | 0.015 | 0.303 | Good | 56.3 | 2 |
| 9 | <i>Niger</i> | ... 0.792 | 1.58 | 10.2 | 0.308 | 0.007 | 0.349 | Good | 49.3 | 15 |
| 8 | <i>White</i> | ... 0.123 | 1.25 | 0.3 | 0.111 | 0.022 | 0.712 | Poor | 51.3 | 14 |
| 4 | <i>Fumigatus</i> | ... 0.015 | 1.12 | 0.4 | 0.096 | 0.010 | 0.278 | Good | 52.0 | 5 |
| 6 | <i>Versicolor</i> | ... 0.015 | 1.29 | nil | 0.125 | 0.026 | 0.290 | Good | 51.8 | 8 |
| 6. | <i>Sydowi</i> | ... 0.028 | 1.25 | -0.2 | 0.149 | 0.017 | 0.175 | Good | 54.1 | 6 |
| 5 | <i>Nidulans</i> (Type B) | ... 0.017 | 1.31 | -0.5 | 0.102 | 0.028 | 0.418 | Good | 51.6 | 4 |
| 2 | <i>Glaucus</i> | ... 0.006 | 1.01 | 3.6 | 0.261 | 0.001 | 0.585 | Poor | 55.4 | 10 |
| 3 | <i>Minimus</i> | ... 0.004 | 1.17 | 3.1 | 0.238 | nil | 0.703 | Good | 54.5 | 2 |
| 10 | <i>Wentii</i> | ... 0.009 | 1.12 | 7.8 | 0.474 | 0.001 | 0.709 | Good | 50.0 | 3 |
| 7 | <i>Ustus</i> | ... 0.003 | 1.12 | 8.8 | 0.527 | — | 0.510 | Good | 51.7 | 1 |

TABLE XV.—Summary of carbon balance sheets for species of *Aspergillus*, arranged according to type of products.
Limiting values.

| No. of Group. | Name of Group. | Carbon in volatile neutral compounds. | Respiration coefficient. | Titration. N/1 acid. | Carbon in non-volatile acids. | Carbon in volatile acids. | Carbon unaccounted for. | Growth on Czapek-Dox metabolism solution. | Carbon in mycelium. | No. of experiments averaged. |
|---------------|--------------------------|---------------------------------------|--------------------------|----------------------|-------------------------------|---------------------------|-------------------------|---|---------------------|------------------------------|
| 1 | <i>Clavatus</i> ... | 2.064-0.594 | 2.48-1.49 | 3.6-0.3 | gm. 0.124-0.059 | gm. 0.022-0.003 | gm. 0.697-0.183 | Good | Per cent. 53.9-47.8 | 6 |
| 11 | <i>Flavus-oryzae</i> ... | 1.483-0.288 | 2.56-1.36 | 6.9 to -0.3 | 0.173-0.090 | 0.050-0.007 | 0.659-0.090 | Good | 53.2-43.5 | 7 |
| 10 | <i>Ochraceus</i> ... | 1.424-0.117 | 3.04-1.42 | 5.1 to -0.1 | 0.145-0.098 | 0.055-0.006 | 1.248-0.240 | Good | 57.8-47.2 | 10 |
| 5 | <i>Nidulans</i> (Type A) | 1.026-0.673 | 1.64-1.62 | 2.4-0.6 | 0.090-0.076 | 0.027-0.020 | 0.798-0.640 | Good | 50.4-49.4 | 3 |
| 7 | <i>Terreus</i> ... | 0.417-0.242 | 1.75-1.48 | 0.8-nil | 0.153-0.133 | 0.024-0.005 | 0.330-0.275 | Good | 56.4-56.1 | 2 |
| 9 | <i>Niger</i> ... | 1.541-0.012 | 1.96-0.99 | 29.3-2.9 | 0.654-0.099 | 0.047-nil | 0.609-0.105 | Good | 52.2-47.1 | 15 |
| 8 | <i>White</i> ... | 0.603-0.004 | 1.58-1.03 | 3.4 to -0.7 | 0.153-0.069 | 0.094-nil | 1.966-0.230 | Poor | 53.4-48.5 | 14 |
| 4 | <i>Fumigatus</i> ... | 0.039-nil | 1.17-1.03 | 1.3 to -0.7 | 0.131-0.069 | 0.031-nil | 0.401-0.145 | Good | 54.5-48.6 | 5 |
| 6 | <i>Versicolor</i> ... | 0.026-0.004 | 1.42-1.14 | 0.3 to -0.7 | 0.217-0.059 | 0.059-0.003 | 0.476 to -0.032 | Good | 54.5-47.5 | 8 |
| 6 | <i>Sydowni</i> ... | 0.150-nil | 1.34-1.12 | 0.9 to -0.9 | 0.178-0.130 | 0.040-nil | 0.400-0.114 | Good | 56.6-52.2 | 6 |
| 5 | <i>Nidulans</i> (Type B) | 0.030-nil | 1.40-1.22 | 0.8 to -1.1 | 0.131-0.080 | 0.043-0.011 | 0.797 to -0.070 | Good | 54.3-46.4 | 4 |
| 2 | <i>Glaucus</i> ... | 0.022-nil | 1.09-0.89 | 5.7-0.6 | 0.469-0.119 | 0.004-nil | 0.948-0.135 | Poor | 59.1-50.9 | 10 |
| 3 | <i>Minimus</i> ... | 0.005-0.002 | 1.19-1.15 | 3.1-3.0 | 0.251-0.224 | nil-nil | 0.791-0.615 | Good | 55.3-53.7 | 2 |
| 10 | <i>Wentii</i> ... | 0.013-0.006 | 1.19-1.03 | 11.5-2.3 | 0.568-0.316 | 0.003-nil | 0.911-0.305 | Good | 51.7-48.8 | 3 |
| 7 | <i>Ustus</i> ... | 0.003 | 1.12 | 8.8 | 0.527 | — | 0.510 | Good | 51.7 | 1 |

Consideration of the results given in Tables XIV and XV, together with detailed examination of the separate balance sheets, lead to two important conclusions :—

- (a) The balance sheets offer a solution of the original aim of this investigation. They enable a choice to be made, from all the species of *Aspergillus* investigated, of those species which may with confidence be subjected to further intensive investigation, since we know from them approximately the yield of product to be expected, and to what type of chemical compound this belongs. Conversely, they indicate which groups to avoid.
- (b) They offer an obvious biochemical classification of species of *Aspergillus* in which these are arranged according to the types of metabolic products arising when the species are grown on glucose.

These two headings will now be dealt with separately, in detail :—

(a) It is evident, in the first place, that all species of the *clavatus*, *flavus-oryzæ*, *ochraceus*, *nidulans* (Type A), *terreus* and *niger* groups give rise to relatively large yields of volatile neutral compounds, in most cases probably ethyl alcohol, while none of this type of compound is to be expected from the *fumigatus*, *versicolor*, *Sydowi*, *nidulans* (Type B), *glaucus*, *minimus*, *Wentii* or *ustus* groups. On the other hand, if search is to be made for organic acids, classed as “ non-volatile acids ” and including all the polybasic acids, these must be sought for in the *niger*, *glaucus*, *minimus*, *Wentii* and *ustus* groups. Outstanding species in this class are *A. cinnamomeus*, *A. niger* (T. and C. 3528.7) and *A. fumaricus* in the *niger* group, *A. Scheelei* and *A. glaucus* in the *glaucus* group, *A. minimus*, *A. Wentii*, strains T. and C. 4202.16 and T. and C. 4230, and *A. ustus*. Up to the present very few species have been investigated by us from the point of view of their production of non-volatile acids. In one case, however, *A. Wentii* T. and C. 4202.16 has been worked upon, largely to determine the nature of the “ carbon unaccounted for,” and it has been shown incidentally that this species gives large yields of gluconic acid (see Part XVII).

Most of the intensive work which it has been found possible to do so far has been carried out with a view to investigating the nature of the products grouped under “ carbon unaccounted for.” Species coming under this heading are scattered over most of the groups, but we have found no strain of *A. fumigatus*, *versicolor*, *Sydowi*, or *terreus* which gives yields of products of this type sufficiently large to be of interest. On the other hand, other species obviously hold out much promise, and this has been fulfilled in those cases where investigation has taken place. Thus, while almost all the white species give large amounts of “ carbon unaccounted for,” species Ac. 56, Ac. 55 and Ac. 10 are pre-eminent in this group with figures of 1.966, 1.771 and 1.181 respectively, corresponding to yields of 48 per cent., 39 per cent. and 28 per cent. of the glucose fermented. These species have been investigated and the results are given in Part IX. Briefly, it may be said that with Ac. 55 and Ac. 10 the “ carbon unaccounted for ” is almost pure mannitol, whilst with Ac. 56 it consists of mannitol and glycerol.

Similarly, strains of *A. nidulans* of both Type A and Type B, with values of "carbon unaccounted for" of about 0.8, give yields of about 20 per cent. of mannitol together with, in the case of Type A strains, an equally large yield of ethyl alcohol. These results are also reported in Part IX.

Though mannitol is of fairly frequent occurrence as a constituent of the "carbon unaccounted for," it does not occur at all in some species, and this supports the belief that intensive examination of chosen representative species will be amply repaid. An example of this type is furnished by the *A. flavus-oryzæ* group. Of this group, *A. parasiticus* SPEARE, Catalogue Number Ac. 14, gives the largest figure for "carbon unaccounted for," i.e., 0.659, corresponding to about 19 per cent. of the glucose fermented. This has been shown to consist almost entirely of a γ -pyrone derivative, somewhat inaccurately called "kojic acid," since it is 5-hydroxy-2-hydroxymethyl- γ -pyrone (see Part VII). The diagnostic value of the carbon balance sheets is evidenced by the fact that, whilst *A. parasiticus* has the highest value for "carbon unaccounted for" and gives the highest yield of kojic acid, this compound is, in fact, produced by all except one species in this group, and in amounts roughly corresponding to the values for "carbon unaccounted for" of these different species.

If it is intended to submit to intensive investigation a number of other selected species of *Aspergillus*, the following are obvious choices:—

Clavatus group.—*A. clavatus* (Washington 138), having 0.697 of "carbon unaccounted for" (18 per cent. of glucose fermented) and 0.835 of "volatile neutral compounds" (21 per cent.).

Glaucus group.—*A. disjunctus*, having 0.948 of "carbon unaccounted for" (31 per cent.) and 0.268 of "non-volatile acids" (9 per cent.).

A. Scheelei, having corresponding figures of 0.752 (27 per cent.) and 0.469 (17 per cent.).

A. minimus, having 0.791 of "carbon unaccounted for" (25 per cent.) and 0.224 of "nonvolatile acids" (7 per cent.).

A. ustus, having 0.510 of "carbon unaccounted for" (12 per cent.) and 0.527 of "non-volatile acids" (13 per cent.).

White group.—Cultures Ac. 48 and Ac. 50, having respective values for "carbon unaccounted for" of 0.672 (26 per cent.) and 0.769 (25 per cent.).

It is obvious, of course, that this is only an arbitrary choice and that the field may be considerably widened if opportunity affords.

(b) It has been stated previously that the carbon balance sheet method offers an obvious biochemical classification of species of *Aspergillus*, in which these are arranged according to the types of metabolic products arising from the growth of the species on glucose. It is not claimed, of course, that this biochemical classification in any way supplants the existing morphological one, but rather that it may be used to supplement the latter. It is claimed, however, that if a carbon balance sheet is prepared for an unnamed species of *Aspergillus* the latter may be placed in its appropriate group

by comparison of its balance sheet with those presented in this report. Thus, if an unnamed species of *Aspergillus* grows poorly on the CZAPEK-Dox solution described, gives rise to no volatile neutral compounds, has a respiration coefficient approximating to unity, gives no volatile acids, but on the contrary gives good yields of non-volatile acids and "carbon unaccounted for," it may with confidence be placed in the *A. glaucus* group. For this reason we regard *A. novus* WEHMER as a member of the *glaucus* group, and since its carbon balance sheet is of an entirely different nature from that of the *flavus-oryzæ* group, we disagree with THOM and CHURCH, who, as stated on p. 30, prefer to regard it as a member of the latter group.

Interesting support has also been given for the biochemical classification in the case of the four cultures Ac. 47, 25, 30 and 18, details of which are given on p. 38. These were purchased from different sources and were supplied as *A. penicillopsis*, *A. Sydowi*, *A. nidulans* and *A. nidulans* respectively. They should belong to Group 11, Group 6 (*Sydowi*) and Group 5 respectively. Their carbon balance sheets, however, unmistakably placed them all in the *versicolor* group (6), with the exception that *A. versicolor* and *A. Sydowi* are not distinguishable by this method. The four cultures were therefore sent to Miss CHURCH, who, on morphological grounds, identified them all as different strains of *A. versicolor*.

Summary.

A quantitative examination has been made by the carbon balance sheet method described in Part II of the types of products formed from glucose by a large number of different species of *Aspergillus* obtained from different parts of the world. These carbon balance sheets are collected in a series of tables arranged in groups. Each group has its own peculiar biochemical characteristics, while the different species in each group have similar characteristics. The grouping of species agrees well with that adopted by THOM and CHURCH on morphological grounds.

By means of the carbon balance sheets a choice has been made of species suitable for intensive examination with a view to isolating and identifying their metabolic products. The results of these investigations are described in subsequent papers.

Studies in the Biochemistry of Micro-organisms.

PART IV.—*Quantitative examination by the carbon balance sheet method of the types of products formed from glucose by species of Penicillium (including Citromyces).*

By JOHN HOWARD BIRKINSHAW, JOHN HENRY VICTOR CHARLES, ARTHUR CLEMENT HETHERINGTON and HAROLD RAISTRICK, with an Appendix by CHARLES THOM.

The work described in Part III of this series, dealing with the preparation of carbon balance sheets for species of *Aspergillus*, was continued on exactly the same lines, using species of *Penicillium* as the organisms for study.

The methods used were exactly the same as those described in Parts II and III, the same medium being used, the same temperature of incubation, 23°–25° C. and the same methods of cultivation and analysis.

The results obtained are given in the following pages and are arranged in groups according to the classification of the *Penicillia* adopted by THOM in his recent book 'The *Penicillia*' (The Williams and Wilkins Co., 1929). The whole of the cultures of *Penicillium*, amounting in all to about a hundred species, were sent to Dr. THOM and were examined by him. In some cases these cultures are described in his book and in all cases Dr. THOM gave his opinion as to the correct names to be applied to the different species. The history of the species used and Dr. THOM's comments on them will be found in an Appendix at the end of this paper. We desire to take this opportunity of thanking Dr. THOM for his very kind co-operation.

GROUP I.

Division I.—Monoverticillata.

Section I. Monoverticillata-stricta.

Subsection 2. Stricta-floccosa.

The species examined in this group (Ad. 74, Ad. 80, Ad. 79, Ad. 78, Ad. 21, Ad. 29, Ad. 71, Ad. 73) consist of eight strains, all of which belong to the *P. (Citromyces) Pfefferianum*–*P. spinulosum* series, and the carbon balance sheets are given in Table I. All these species would formerly have been included in WEHMER's genus *Citromyces*, but, as the modern tendency is to eliminate this genus, the procedure adopted by Dr. THOM is followed in this paper.

TABLE I.

| Species of <i>Penicillium</i> : | | <i>P. (Clitomyces) Pfefferianum-P. spinulosum</i> series. | | | | | | | | |
|--|-----------|---|--------|--------|--------|--------|--------|--------|--------|--|
| Catalogue number : | | Ad. 74 | Ad. 80 | Ad. 79 | Ad. 78 | Ad. 21 | Ad. 29 | Ad. 71 | Ad. 73 | |
| Experiment number : | | F 55 | F 64 | F 63 | F 59 | 126 | F 11 | F 78 | F 82 | |
| Incubation period in days : | | 77 | 72 | 70 | 61 | 37 | 64 | 40 | 70 | |
| <i>Carbon Balance Sheet.</i> | | | | | | | | | | |
| Carbon in solution (start) ... | gm. | 4.944 | 4.952 | 4.952 | 4.944 | 5.043 | 4.944 | 4.952 | 4.952 | |
| Carbon in H ₂ SO ₄ ... | " | 0.002 | 0.002 | 0.001 | 0.002 | 0.001 | — | 0.001 | 0.002 | |
| " in CO ₂ ... | " | — | 2.203 | 1.847 | 2.235 | 1.294 | 2.046 | 1.416 | 1.884 | |
| " in mycelium ... | " | 0.887 | 0.747 | 0.661 | 0.834 | 0.642 | 0.821 | 0.551 | 0.707 | |
| " in solution (end) ... | " | 1.655 | 1.885 | 2.423 | 1.735 | 2.952 | 2.004 | 2.843 | 2.150 | |
| " accounted for ... | " | — | 4.837 | 4.932 | 4.806 | 4.889 | 4.871 | 4.811 | 4.743 | |
| " accounted for ... | per cent. | — | 97.7 | 99.6 | 97.2 | 97.0 | 98.5 | 97.2 | 95.8 | |
| <i>Analysis of Solution.</i> | | | | | | | | | | |
| Carbon in residual glucose ... | gm. | 0.252 | 0.577 | 1.136 | 0.689 | 2.014 | 1.106 | 1.935 | 1.217 | |
| " in CO ₂ in solution ... | " | nil | 0.002 | nil | 0.001 | 0.003 | 0.007 | 0.009 | 0.002 | |
| " in volatile acids ... | " | 0.002 | 0.009 | nil | 0.009 | 0.011 | 0.027 | 0.027 | 0.034 | |
| " in non-volatile acids ... | " | 0.937 | 0.799 | 0.862 | 0.358 | 0.314 | 0.318 | 0.318 | 0.285 | |
| " in volatile neutral compounds ... | " | 0.036 | 0.083 | 0.032 | 0.014 | nil | 0.060 | 0.060 | 0.033 | |
| " in synthetic compounds ... | " | 0.212 | 0.135 | 0.069 | 0.085 | 0.211 | 0.123 | 0.170 | 0.111 | |
| Total carbon accounted for ... | " | 1.439 | 1.605 | 2.099 | 1.156 | 2.553 | 1.641 | 2.519 | 1.682 | |
| " " in solution ... | " | 1.655 | 1.885 | 2.423 | 1.735 | 2.952 | 2.004 | 2.843 | 2.150 | |
| Carbon unaccounted for (by difference) ... | " | 0.216 | 0.280 | 0.324 | 0.579 | 0.399 | 0.363 | 0.324 | 0.468 | |
| <i>Residual Glucose.</i> | | | | | | | | | | |
| Glucose (by polarimeter) ... | per cent. | 0.099 | 0.287 | 0.623 | 0.430 | 1.035 | 0.521 | 1.051 | 0.583 | |
| " (SHAFFER-HARTMANN) ... | " | 0.126 | 0.288 | 0.568 | 0.345 | 1.007 | 0.553 | 0.968 | 0.608 | |
| " (WOOD-OST) ... | " | — | — | 0.571 | — | 1.066 | 0.571 | 0.930 | — | |
| " (by alkaline iodine)... | " | 0.144 | 0.317 | 0.655 | 0.463 | 1.111 | 0.569 | 1.049 | 0.676 | |
| <i>Acids.</i> | | | | | | | | | | |
| Titration (N/1 acid) ... | c.c. | 29.1 | 21.7 | 20.3 | 5.0 | 5.1 | 5.7 | 5.2 | 4.4 | |
| Volatile acids (N/1 acid) ... | " | 0.17 | 0.83 | 0.03 | 0.74 | 1.51 | 1.01 | 1.47 | 1.43 | |
| Barium salts (weight) ... | gm. | 0.029 | 0.048 | 0.015 | 0.041 | 0.069 | 0.102 | 0.110 | 0.152 | |
| Calcium salts (weight) ... | " | 3.474 | 2.958 | 2.957 | 1.327 | 1.460 | 1.625 | 1.250 | 1.248 | |
| Volume of oxygen absorbed | " | — | 3865 | 3289 | 3460 | 2059 | 3406 | 2326 | 3272 | |
| Respiration coefficient ... | c.c. | — | 1.06 | 1.05 | 1.21 | 1.18 | 1.13 | 1.14 | 1.08 | |
| Mycelium (weight) ... | gm. | 1.726 | 1.427 | 1.298 | 1.707 | 1.276 | 1.644 | 1.069 | 1.355 | |
| " (carbon) ... | per cent. | 51.4 | 52.4 | 50.9 | 48.8 | 50.3 | 49.9 | 51.6 | 52.2 | |

The whole of these species form a well-defined group having certain very definite characteristics :—

- (i) All give rise to large amounts of non-volatile acids. This is particularly marked in the case of strains Ad. 74, Ad. 80 and Ad. 79, which give larger amounts of titratable acidity and non-volatile acids than any other species of *Penicillium* examined. The nature of this acidity has been investigated, has been proved to be due to citric acid, and is reported in Part XII of this series. These species are also distinguished by the production of a purple-coloured substance, a methoxy-dihydroxy-toluquinone, which is also described in Part XII. The remaining five species give rise to smaller, but still quite considerable, quantities of titratable acidity and non-volatile acids.
- (ii) None of the eight strains produces appreciable amounts of alcohol ("carbon in volatile neutral compounds"), though, with the exception of Ad. 21, all of them give rise to detectable amounts. A natural sequence to this is the fact that all these strains have low respiration coefficients.
- (iii) All the strains give moderate amounts of "carbon unaccounted for."

GROUP II.

Division I.—Monoverticillata.

Section I. Monoverticillata-stricta.

Subsection 4. Velutina.

This group includes five strains (Ad. 6, Ad. 7, Ad. 67, Ad. 68 and Ad. 69) which fall in the *P. (Citromyces) glabrum* WEHMER series, and one strain (Ad. 48) which has been diagnosed as *P. aurantio-violaceum* BOURGE. The carbon balance sheets for these species are given in Table II.

Formerly the five strains would have been included in WEHMER's genus *Citromyces* as strains of *Citromyces glaber*, but, as explained under Group I, they are now classed as species of *Penicillium*. These five strains form a well-defined group and are of especial interest since all of them give rise to a new biochemical product, which is specific for this group, to which we propose to give the name "citromycetin" and which is described in detail in Part XI of this series. In contradistinction, none of the strains in Group I produces this substance. In addition to this specific product, all the five strains of *P. (Citromyces) glabrum* also show the following characteristics :—

- (i) All give rise to considerable amounts of titratable acidity in the form of non-volatile acids. This has been shown to be citric acid, though, in the case of cultures incubated under full aerobic conditions, *i.e.*, in flasks plugged with cotton-wool, a portion of the acidity is caused by the presence of citromycetin. The last carbon balance sheet in Table II (Expt. No. 143) was prepared from a

TABLE II.

| Species of <i>Penicillium</i> : | <i>P. aurantio-violaceum</i> BOURGE. | <i>P. (Citromyces) glabrum</i> WEHMER series. | | | | | |
|---|--------------------------------------|---|-------|--------|--------|--------|------------------|
| | | | | | | | |
| Catalogue number : | Ad. 48 | Ad. 6 | Ad. 7 | Ad. 67 | Ad. 68 | Ad. 69 | Ad. 7 |
| Experiment number : | F 36 | 134 | F 5 | F 74 | F 75 | F 76 | 143 |
| Incubation period in days : | 68 | 51 | 55 | 19 | 32 | 42 | 10 |
| <i>Carbon Balance Sheet.</i> | | | | | | | |
| Carbon in solution (start) gm. | 4.944 | 5.043 | 5.043 | 4.952 | 4.952 | 4.952 | 4.944 |
| Carbon in H ₂ SO ₄ " | 0.001 | 0.003 | 0.003 | 0.003 | 0.002 | 0.001 | — |
| " in CO ₂ " | 2.385 | 2.008 | 2.277 | 1.292 | 1.150 | 1.524 | — |
| " in mycelium " | 1.280 | 0.872 | 1.030 | 0.692 | 0.462 | 0.582 | 1.339 |
| " in solution (end) " | 1.174 | 1.973 | 1.843 | 2.842 | 3.258 | 2.765 | 1.588 |
| " accounted for " | 4.840 | 4.856 | 5.153 | 4.829 | 4.872 | 4.872 | — |
| " accounted for per cent. | 97.9 | 96.3 | 102.2 | 97.5 | 98.4 | 98.4 | — |
| <i>Analysis of Solution.</i> | | | | | | | |
| Carbon in residual glucose gm. | 0.848 | 0.663 | 0.536 | 1.578 | 2.348 | 1.681 | 0.912 |
| " in CO ₂ in solution " | 0.015 | 0.001 | 0.004 | 0.004 | 0.003 | 0.002 | nil |
| " in volatile acids... .. " | 0.015 | 0.026 | 0.016 | 0.025 | 0.021 | 0.039 | 0.001 |
| " in non-volatile acids " | 0.154 | 0.345 | 0.402 | 0.304 | 0.341 | 0.315 | 0.556 |
| " in volatile neutral compounds " | 0.006 | 0.126 | 0.101 | 0.208 | 0.059 | 0.071 | 0.013 |
| " in synthetic compounds " | 0.083 | 0.191 | 0.218 | 0.036 | 0.093 | 0.090 | 0.397 |
| Total carbon accounted for " | 1.121 | 1.352 | 1.277 | 2.155 | 2.865 | 2.198 | 1.879 |
| " " in solution... .. " | 1.174 | 1.973 | 1.843 | 2.842 | 3.258 | 2.765 | 1.588 |
| Carbon unaccounted for (by difference) .. | 0.053 | 0.621 | 0.566 | 0.687 | 0.393 | 0.567 | Surplus of 0.291 |
| <i>Residual Glucose.</i> | | | | | | | |
| Glucose (by polarimeter) per cent. | 0.459 | 0.340 | 0.301 | 0.856 | 1.342 | 0.969 | 0.331 |
| " (SHAFFER-HARTMANN) " | 0.424 | 0.331 | 0.268 | 0.789 | 1.174 | 0.841 | 0.456 |
| " (WOOD-OST) " | 0.435 | — | — | 0.815 | 1.178 | 0.838 | 0.454 |
| " (by alkaline iodine) " | 0.430 | 0.397 | 0.339 | 0.899 | 1.302 | 0.926 | 1.177 |
| <i>Acids.</i> | | | | | | | |
| Titration (N/1 acid) c.c. | 0.3 | 8.4 | 6.6 | 4.1 | 7.6 | 7.9 | 9.2 |
| Volatile acids (N/1 acid) " | 0.89 | 1.72 | 1.48 | 1.48 | 1.47 | 1.79 | 0.17 |
| Barium salts (weight) gm. | 0.101 | 0.089 | 0.068 | 0.140 | 0.089 | 0.189 | 0.014 |
| Calcium salts (weight) " | 0.674 | 1.667 | 1.597 | 1.117 | 1.559 | 1.280 | 1.819 |
| Volume of oxygen absorbed c.c. | 4016 | 3225 | 3277 | 1854 | 1878 | 2501 | — |
| Respiration coefficient | 1.11 | 1.16 | 1.30 | 1.27 | 1.15 | 1.14 | — |
| Mycelium (weight) gm. | 2.542 | 1.754 | 2.054 | 1.364 | 0.919 | 1.124 | 2.518 |
| " (carbon) per cent. | 50.4 | 49.7 | 50.2 | 50.7 | 50.3 | 51.7 | 53.2 |

culture grown under these conditions and is incorporated here as illustrating the tremendous effect which full aeration produces on the type of metabolic products formed.

- (ii) All strains give rise to small but definite amounts of volatile neutral compounds and have in consequence small respiration coefficients.
- (iii) All strains give rise to marked amounts of "carbon unaccounted for."

P. aurantio-violaceum BOURGE, Ad. 48, gives a carbon balance sheet which is so different in type from the other carbon balance sheets in Table II that, from a biochemical point of view, it cannot logically be placed in this group. Physiological tests also show this species to be quite different from the other strains.

GROUP III.

Division II.—Asymmetrica.

Section I. Velutina.

Subsection 1. Velutina-elliptica-magna.

The species examined in this group (Ad. 52, Ad. 81 and Ad. 102) consists of three strains of *P. digitatum* SACCARDI, obtained from different parts of the world. The carbon balance sheets are given in Table III. The most striking feature of these carbon balance sheets, which are all of the same type, is the relatively large figure obtained for "carbon in H_2SO_4 ." The figure obtained with these three strains is about ten times as large as that obtained with any other species in any genus examined. It is shown in Part XVIII of this series that the substance responsible for this high figure is ethyl acetate, and this species appears to be unique in the production, at any rate in considerable quantities, of this ester. The production of this ester is further indicated by the fact that cultures of this species, grown under the conditions of the metabolism experiments, have a very agreeable ester smell.

Further points of interest in the carbon balance sheets are (a) the large amounts of "carbon in volatile neutral compounds," which have been shown to be due to ethyl alcohol (see Part XVIII), and (b) the relatively high respiration coefficient.

TABLE III.

| Species of <i>Penicillium</i> : | | <i>P. digitatum</i> SACCARDO. | | | |
|--|--|-------------------------------|--------|--------|---------|
| Catalogue number : | | Ad. 52 | Ad. 52 | Ad. 81 | Ad. 102 |
| Experiment number : | | F 40 | F 60 | F 86 | F 87 |
| Incubation period in days : | | 81 | 58 | 88 | 80 |
| <i>Carbon Balance Sheet.</i> | | | | | |
| Carbon in solution (start) gm. | | 4.944 | 4.944 | 4.719 | 4.719 |
| „ in H ₂ SO ₄ „ | | 0.177 | 0.207 | 0.072 | 0.082 |
| „ in CO ₂ „ | | 2.601 | 2.080 | 1.652 | 2.254 |
| „ in mycelium „ | | 0.530 | 0.604 | 0.412 | 0.436 |
| „ in solution (end) „ | | 1.395 | 1.917 | 2.404 | 1.918 |
| „ accounted for „ | | 4.703 | 4.808 | 4.540 | 4.690 |
| „ accounted for per cent. | | 95.1 | 97.2 | 96.2 | 99.4 |
| <i>Analysis of Solution.</i> | | | | | |
| Carbon in residual glucose gm. | | 0.038 | 0.314 | 0.649 | 0.030 |
| „ in CO ₂ in solution „ | | 0.007 | 0.010 | 0.012 | 0.006 |
| „ in volatile acids „ | | 0.007 | 0.018 | 0.029 | 0.007 |
| „ in non-volatile acids „ | | 0.107 | 0.098 | 0.079 | 0.104 |
| „ in volatile neutral compounds „ | | 0.968 | 0.992 | 1.043 | 1.416 |
| „ in synthetic compounds „ | | 0.066 | 0.013 | 0.138 | 0.026 |
| Total carbon accounted for „ | | 1.193 | 1.445 | 1.950 | 1.589 |
| „ „ in solution „ | | 1.395 | 1.917 | 2.404 | 1.918 |
| Carbon unaccounted for (by difference) .. | | 0.202 | 0.472 | 0.454 | 0.329 |
| <i>Residual Glucose.</i> | | | | | |
| Glucose (by polarimeter) per cent. | | 0.015 | 0.131 | 0.393 | 0.032 |
| „ (SHAFFER-HARTMANN) „ | | 0.019 | 0.157 | — | — |
| „ (WOOD-OST) „ | | — | — | 0.325 | 0.015 |
| „ (by alkaline iodine) „ | | 0.070 | 0.182 | 0.401 | 0.082 |
| <i>Acids.</i> | | | | | |
| Titration (N/1 acid) c.c. | | 0.7 | 1.4 | 3.1 | nil |
| Volatile acids (N/1 acid) „ | | 1.62 | 0.76 | 0.08 | nil |
| Barium salts (weight) gm. | | 0.152 | 0.087 | 0.127 | 0.077 |
| Calcium salts (weight) „ | | 0.436 | 0.415 | 0.390 | 0.317 |
| Volume of oxygen absorbed c.c. | | 3492 | 2710 | 1904 | 2730 |
| Respiration coefficient „ | | 1.39 | 1.44 | 1.63 | 1.55 |
| Mycelium (weight) gm. | | 1.086 | 1.262 | 0.919 | 0.966 |
| „ (carbon) per cent. | | 50.0 | 47.8 | 44.8 | 45.1 |

GROUP IV.

*Division II.—Asymmetrica.**Section I. Velutina.**Subsection 2. Velutina-divaricata.*

Only two species are included in this group, *i.e.*, Ad. 42 (*P. Steckii* ZALESKI) and Ad. 23 (*P. citrinum* THOM). The carbon balance sheets for these species, given in Table IV, show the following characteristics:—

- (i) Moderate amounts of titratable acidity and “carbon in non-volatile acids.”
- (ii) Negligible amounts of “carbon in volatile neutral compounds” and hence low respiration coefficients.
- (iii) Considerable amounts of “carbon unaccounted for.”

Ad. 42 thus gives a very similar carbon balance sheet to Ad. 23 and it has also been shown to have very similar physiological properties, but Ad. 23 (*P. citrinum* THOM) is distinguished from Ad. 42, and indeed from all other species examined, either of *Penicillium* or of any other genus, by the fact that it and two other strains of *P. citrinum* (Ad. 95 and Ad. 114) have been shown to produce a specific biochemical product, to which the name citrinin has been given (see Parts XIV and XV).

GROUP V.

*Division II.—Asymmetrica.**Section I. Velutina.**Subsection 3. Radiata (P. chrysogenum series).*

Included in this group are six species (Ad. 11, Ad. 24, Ad. 56, Ad. 14, Ad. 35 and Ad. 53) which are all closely related morphologically, biochemically and physiologically. The carbon balance sheets, which are given in Table V, show the following characteristics:—

- (i) With one exception, Ad. 24, they all give rise to exceptionally large amounts of “carbon unaccounted for,” amounting in one case, Ad. 11, to over 30 per cent. of the glucose metabolized. The nature of the metabolic products of Ad. 11 has been investigated and is reported in detail in Part XVII of this series. Suffice it to say here that the “carbon unaccounted for” consists, in this case, principally of mannitol.
- (ii) All the species, again with the exception of Ad. 24, show large amounts of titratable acidity and high figures for “carbon in non-volatile acids.”
- (iii) None of the species forms alcohol except in very small amounts and in consequence all of them have low respiration coefficients.

The species in this group as a whole seem to stand out from the majority of species of *Penicillium* because of the large amounts of total metabolic products other than CO₂ and alcohol which they form.

TABLE IV.

| Species of <i>Penicillium</i> : | <i>P. Steckii</i> ZALESKI. | <i>P. citrinum</i> THOM. |
|---|----------------------------|--------------------------|
| Catalogue number : | Ad. 42 | Ad. 23 |
| Experiment number : | F 30 | F 23 |
| Incubation period in days : | 58 | 43 |
| <i>Carbon Balance Sheet.</i> | | |
| Carbon in solution (start) gm | 4.944 | 4.944 |
| Carbon in H ₂ SO ₄ " | 0.001 | 0.001 |
| " in CO ₂ " | 1.780 | 1.443 |
| " in mycelium " | 1.080 | 0.629 |
| " in solution (end) " | 1.992 | 2.804 |
| " accounted for " | 4.853 | 4.877 |
| " accounted for per cent. | 98.2 | 98.6 |
| <i>Analysis of Solution.</i> | | |
| Carbon in residual glucose gm. | 0.770 | 2.026 |
| " in CO ₂ in solution " | 0.001 | 0.009 |
| " in volatile acids " | 0.002 | 0.024 |
| " in non-volatile acids " | 0.268 | 0.202 |
| " in volatile neutral compounds " | 0.004 | 0.048 |
| " in synthetic compounds " | 0.160 | 0.053 |
| Total carbon accounted for " | 1.205 | 2.362 |
| " " in solution " | 1.992 | 2.804 |
| Carbon unaccounted for (by difference) " | 0.787 | 0.442 |
| <i>Residual Glucose.</i> | | |
| Glucose (by polarimeter) per cent. | 0.516 | 1.033 |
| " (SHAFFER-HARTMANN) " | 0.385 | 1.013 |
| " (WOOD-OST) " | 0.386 | 1.020 |
| " (by alkaline iodine) " | 0.433 | 1.116 |
| <i>Acids.</i> | | |
| Titration (N/1 acid) c.c. | 3.3 | 3.0 |
| Volatile acids (N/1 acid) " | nil | 1.18 |
| Barium salts (weight) gm. | 0.004 | 0.112 |
| Calcium salts (weight) " | 0.930 | 0.869 |
| Volume of oxygen absorbed c.c. | 2902 | 2212 |
| Respiration coefficient " | 1.15 | 1.23 |
| Mycelium (weight) gm. | 2.280 | 1.176 |
| " (carbon) per cent. | 47.3 | 53.5 |

TABLE V.

| Species of <i>Penicillium</i> : | | | <i>P. chrysogenum</i> series. | | | | <i>P. breviculatum</i> WESTLING. | <i>P. notatum</i> WESTLING. | <i>P. melaleucum</i> BIOURGET. | <i>P. puberulum</i> BAINIER. | <i>P. Boursget-anum</i> ZALESKI. |
|--|--------|--------|-------------------------------|--------|--------|--------|-------------------------------------|--------------------------------|-----------------------------------|---------------------------------|-------------------------------------|
| | | | <i>P. chrysogenum</i> series. | | | | | | | | |
| Catalogue number : | Ad. 11 | Ad. 24 | Ad. 56 | Ad. 14 | Ad. 35 | Ad. 53 | Ad. 31 | Ad. 87 | | | |
| Experiment number : | F 66 | F 21 | F 44 | F 68 | F 20 | F 43 | F 13 | F 85 | | | |
| Incubation period in days : | 47 | 64 | 48 | 54 | 69 | 56 | 48 | 43 | | | |
| <i>Carbon Balance Sheet.</i> | | | | | | | | | | | |
| Carbon in solution (start) ... | 4.952 | 4.944 | 4.944 | 4.952 | 4.944 | 4.944 | 4.944 | 4.952 | | | |
| Carbon in H ₂ SO ₄ ... | 0.001 | 0.001 | nil | 0.001 | 0.001 | 0.001 | 0.012 | 0.002 | | | |
| " in CO ₂ ... | 1.675 | 2.241 | 1.563 | 1.838 | 1.784 | 1.790 | 1.749 | 1.862 | | | |
| " in mycelium ... | 1.024 | 1.574 | 1.003 | 0.923 | 0.998 | 0.937 | 0.696 | 0.858 | | | |
| " in solution (end) ... | 2.176 | 0.987 | 2.272 | 2.107 | 2.155 | 2.154 | 2.397 | 2.143 | | | |
| " accounted for ... | 4.876 | 4.803 | 4.838 | 4.869 | 4.938 | 4.882 | 4.854 | 4.865 | | | |
| " accounted for ... | 98.5 | 97.1 | 97.8 | 98.3 | 99.9 | 98.8 | 98.2 | 98.2 | | | |
| <i>Analysis of Solution.</i> | | | | | | | | | | | |
| Carbon in residual glucose ... | 0.069 | 0.278 | 0.774 | 0.662 | 0.116 | 0.076 | 0.264 | 0.371 | | | |
| " in CO ₂ in solution ... | nil | 0.012 | nil | 0.001 | nil | 0.002 | 0.003 | nil | | | |
| " in volatile acids ... | nil | 0.018 | nil | 0.003 | 0.001 | 0.008 | 0.006 | 0.006 | | | |
| " in non-volatile acids ... | 0.520 | 0.247 | 0.345 | 0.266 | 0.816 | 0.613 | 0.300 | 0.423 | | | |
| " in volatile neutral compounds ... | 0.004 | 0.014 | 0.001 | 0.013 | 0.017 | 0.013 | 0.863 | 0.021 | | | |
| " in synthetic compounds ... | 0.089 | 0.081 | 0.078 | 0.235 | 0.061 | 0.106 | 0.076 | 0.075 | | | |
| Total carbon accounted for ... | 0.682 | 0.650 | 1.198 | 1.180 | 1.011 | 0.818 | 1.506 | 0.896 | | | |
| " " in solution ... | 2.176 | 0.987 | 2.272 | 2.107 | 2.155 | 2.154 | 2.397 | 2.143 | | | |
| Carbon unaccounted for (by difference) ... | 1.494 | 0.337 | 1.074 | 0.927 | 1.144 | 1.336 | 0.891 | 1.247 | | | |
| <i>Residual Glucose.</i> | | | | | | | | | | | |
| Glucose (by polarimeter) ... | 0.060 | 0.108 | 0.518 | 0.406 | 0.263 | 0.200 | 0.222 | 0.324 | | | |
| " (SHAFFER-HARTMANN) ... | 0.035 | 0.139 | 0.387 | 0.331 | 0.058 | 0.038 | 0.132 | 0.186 | | | |
| " (WOOD-OST) ... | — | — | 0.406 | — | — | — | — | — | | | |
| " (by alkaline iodine) ... | 0.091 | 0.270 | 0.448 | 0.392 | 0.082 | 0.072 | 0.172 | 0.202 | | | |
| <i>Acids.</i> | | | | | | | | | | | |
| Titration (N/1 acid) ... | 8.2 | 1.7 | 5.9 | 3.1 | 11.7 | 11.2 | 3.5 | 7.8 | | | |
| Volatile acids (N/1 acid) ... | nil | 0.85 | ni | nil | nil | 0.80 | nil | 1.91 | | | |
| Barium salts (weight) ... | 0.018 | 0.143 | 0.034 | 0.015 | 0.067 | 0.042 | 0.016 | 0.044 | | | |
| Calcium salts (weight) ... | 1.803 | 1.230 | 1.118 | 0.776 | 2.739 | 1.963 | 1.073 | 1.457 | | | |
| Volume of oxygen absorbed | 2851 | 3317 | 2564 | 3070 | 3203 | 2696 | 2078 | 2927 | | | |
| Respiration coefficient ... | 1.10 | 1.27 | 1.14 | 1.12 | 1.04 | 1.24 | 1.57 | 1.16 | | | |
| Mycelium (weight) ... | 2.151 | 2.690 | 2.116 | 1.915 | 1.845 | 1.891 | 1.438 | 1.662 | | | |
| " (carbon) ... | 47.6 | 58.5 | 47.4 | 48.2 | 54.1 | 49.5 | 48.4 | 51.6 | | | |

GROUP VI.

*Division II.—Asymmetrica.**Section I. Velutina.**Subsection 4. Velutina-restricta.*

Only one species in this subsection was investigated and the carbon balance sheet is given in Table V.

This species, Ad. 31, *P. puberulum* BAINIER, is the one from cultures of which ALSBERG and BLACK isolated penicillic acid. The balance sheet shows relatively large amounts of both "carbon in volatile neutral compounds" and "carbon unaccounted for," together with smaller amounts of titratable acidity and "carbon in non-volatile acids." The metabolic products of this species are at present under investigation.

GROUP VII.

*Division II.—Asymmetrica.**Section II. Brevi-compacta.*

The only species included in this group which was examined was Ad. 87, *P. Biourgeianum* ZALESKI. Its carbon balance sheet is given in Table V and is of the same type as the carbon balance sheets of species in the subsection *Radiata* (*P. chrysogenum* series) also given in Table V. It is characterized by the large amounts of "carbon unaccounted for," the relatively high titratable acidity present in the form of "non-volatile acids" and the absence of appreciable amounts of "volatile neutral compounds."

GROUP VIII.

*Division II.—Asymmetrica.**Section III. Lanata-typica.*

Seven species (Ad. 25, Ad. 49, Ad. 34, Ad. 46, Ad. 12, Ad. 33 and Ad. 60) are included in this group and the carbon balance sheets are given in Table VI. Ad. 25 and Ad. 49, which are different strains of *P. caseicolum* BAINIER, and Ad. 34, *P. Camemberti* THOM, are species isolated from cheese and form a well-defined sub-group having the following characteristics: the carbon balance sheets are of the same type and in no case is any metabolic product other than CO₂ formed in appreciable amounts. The other four species, Ad. 46 (*P. ochraceum* THOM), Ad. 12 (*P. lanoso-viride* THOM), Ad. 33 (*P. lanosum* WESTLING) and Ad. 60 (*P. lanoso-cæruleum* THOM), form a sub-group having certain characteristics in common but showing certain obvious differences. Thus, Ad. 46 and Ad. 12 give rise to large amounts of "volatile neutral compounds," of "carbon unaccounted for," have large respiration coefficients, but do not produce appreciable amounts of titratable acidity, either of a volatile or non-volatile nature. Ad. 60 agrees with the first two species in producing considerable amounts of "volatile

TABLE VI.

| Species of <i>Penicillium</i> : | <i>P. caseicolum</i> BAINIER. | | <i>P.</i> <i>Camemberti</i> THOM. | <i>P.</i> <i>ochraceum</i> THOM. | <i>P.</i> <i>lanoso-</i> <i>viride</i> THOM. | <i>P.</i> <i>lanosum</i> WESTLING. | <i>P.</i> <i>lanoso-</i> <i>cæruleum</i> THOM. |
|---|----------------------------------|--------|---|--|---|--|---|
| Catalogue number : | Ad. 25 | Ad. 49 | Ad. 34 | Ad. 46 | Ad. 12 | Ad. 33 | Ad. 60 |
| Experiment number : | F 16 | F 37 | F 19 | F 34 | F 67 | F 15 | F 48 |
| Incubation period in days : | 41 | 69 | 61 | 57 | 37 | 50 | 67 |
| <i>Carbon Balance Sheet.</i> | | | | | | | |
| Carbon in solution (start) gm. | 4.944 | 4.944 | 4.944 | 4.944 | 4.952 | 4.944 | 4.944 |
| Carbon in H ₂ SO ₄ " | 0.001 | 0.001 | 0.001 | lost | 0.020 | 0.001 | 0.036 |
| " in CO ₂ " | 1.255 | 2.142 | 2.414 | 1.900 | 1.988 | 1.540 | 2.558 |
| " in mycelium " | 0.452 | 0.576 | 0.730 | 0.776 | 0.575 | 1.015 | 1.029 |
| " in solution (end)... .. " | 3.192 | 2.165 | 1.726 | 2.063 | 2.357 | 2.352 | 1.193 |
| " accounted for " | 4.900 | 4.884 | 4.871 | 4.739 | 4.940 | 4.908 | 4.816 |
| " accounted for per cent. | 99.1 | 98.8 | 98.5 | 95.8 | 99.8 | 99.3 | 97.4 |
| <i>Analysis of Solution.</i> | | | | | | | |
| Carbon in residual glucose gm. | 2.914 | 1.718 | 1.398 | 0.048 | 0.128 | 1.008 | 0.012 |
| " in CO ₂ in solution " | 0.017 | 0.005 | 0.012 | 0.002 | 0.003 | 0.001 | 0.017 |
| " in volatile acids " | 0.014 | nil | 0.005 | 0.003 | 0.001 | nil | 0.001 |
| " in non-volatile acids " | 0.109 | 0.321 | 0.186 | 0.299 | 0.142 | 0.365 | 0.168 |
| " in volatile neutral compounds " | 0.031 | 0.007 | 0.017 | 1.053 | 1.301 | 0.006 | 0.675 |
| " in synthetic compounds " | 0.056 | 0.071 | 0.078 | 0.164 | 0.031 | 0.078 | 0.138 |
| Total carbon accounted for " | 3.141 | 2.122 | 1.696 | 1.569 | 1.606 | 1.458 | 1.011 |
| " " in solution " | 3.192 | 2.165 | 1.726 | 2.063 | 2.357 | 2.352 | 1.193 |
| Carbon unaccounted for (by difference) .. | 0.051 | 0.043 | 0.030 | 0.494 | 0.751 | 0.894 | 0.182 |
| <i>Residual Glucose.</i> | | | | | | | |
| Glucose (by polarimeter) per cent. | 1.501 | 0.929 | 0.725 | 0.104 | 0.114 | 0.674 | — |
| " (SHAFFER-HARTMANN) " | 1.457 | 0.859 | 0.699 | 0.024 | 0.064 | 0.504 | 0.006 |
| " (WOOD-OST) " | 1.479 | 0.914 | 0.710 | — | — | 0.519 | — |
| " (by alkaline iodine) " | 1.504 | 0.928 | 0.731 | 0.066 | 0.126 | 0.527 | 0.064 |
| <i>Acids.</i> | | | | | | | |
| Titration (N/1 acid) c.c. | 0.2 | 0.4 | 0.1 | 2.0 | 0.3 | 4.2 | Decrease of 0.7 |
| Volatile acids (N/1 acid) " | 0.93 | 0.28 | 0.52 | nil | 0.08 | nil | 0.77 |
| Barium salts (weight) gm. | 0.060 | 0.001 | 0.016 | 0.044 | 0.017 | 0.016 | 0.047 |
| Calcium salts (weight) " | 1.145 | 1.621 | 0.767 | 1.068 | 0.492 | 1.231 | 0.637 |
| Volume of oxygen absorbed c.c. | 2122 | 3654 | 4203 | 2145 | 2203 | 2533 | 3966 |
| Respiration coefficient " | 1.12 | 1.12 | 1.08 | 1.65 | 1.69 | 1.14 | 1.21 |
| Mycelium (weight) gm. | 0.940 | 1.205 | 1.542 | 1.620 | 1.229 | 2.107 | 2.113 |
| " (carbon) per cent. | 48.1 | 46.3 | 47.3 | 47.9 | 46.8 | 48.2 | 48.7 |

neutral compounds" and in the non-formation of acidity. On the other hand, this species gives rise to only small amounts of "carbon unaccounted for." Ad. 33 agrees with Ad. 46 and Ad. 12 in giving a large amount of "carbon unaccounted for," but differs from them in giving a moderate amount of "non-volatile acids." in the complete absence of "volatile neutral compounds" and in having a respiration coefficient approximating to unity. It has also been found that these four species differ materially in certain physiological details. There is thus no evidence that they form a group of species which are closely related biochemically.

Morphological details of Ad. 12 are given in THOM'S '*Penicillia*' on p. 314, and of Ad. 33 on p. 317.

GROUP IX.

Division II.—Asymmetrica.

Section III. Lanata-typica.

Subsection 2. Lanata-zonata.

This group includes two species, Ad. 82 (*P. commune* THOM) and Ad. 19 (*P. fusco-glaucum* BIOURGE), the carbon balance sheets for which are given in Table VII. These carbon balance sheets, which are very similar in characteristics, are of the same type as those given in Table VI for Ad. 46 and Ad. 12. This is, in fact, so much the case, that it is difficult to distinguish between the balance sheets of Ad. 19 and Ad. 12 and from this point of view these two species might quite well be included biochemically in the same group as Ad. 46 and Ad. 12.

Morphological details of Ad. 19 are given in THOM'S '*Penicillia*' on p. 326.

GROUP X.

Division II.—Asymmetrica.

Section IV. Lanata-divaricata.

Two different strains of *P. lilacinum* THOM, obtained from different parts of the world, are included in this group, their carbon balance sheets being given in Table VII. Morphologically and physiologically these two strains agree well with each other, but consideration of the two carbon balance sheets shows that these are of different types and it appears that these two strains show the same biochemical differences as has previously been noted in Part III with strains of *Aspergillus nidulans* (p. 35). Thus the carbon balance sheet of the strain Ad. 32 is of the same general type as that of the four strains *A. nidulans*, Ac. 67, Ac. 78, Ac. 84 and Ac. 85, while the strain Ad. 37 is of the type represented by *A. nidulans*, Ac. 80 (see Part III, Table V). The main difference between these two strains is the production by Ad. 32 of considerable amounts of "volatile neutral compounds," while Ad. 37 produces only negligible amounts.

TABLE VII.

| Species of <i>Penicillium</i> : | <i>P. commune</i> THOM. | <i>P. fusco-</i> <i>glaucum</i> BIOURGE. | <i>P. lilacinum</i> THOM. | | <i>P. Daleæ</i> ZALESKI. | <i>P. Godlewskii</i> ZALESKI. |
|---|----------------------------|--|---------------------------|---------------------|-----------------------------|----------------------------------|
| Catalogue number : | Ad. 82 | Ad. 19 | Ad. 32 | Ad. 37 | Ad. 55 | Ad. 62 |
| Experiment number : | F 79 | F 72 | F 14 | F 25 | F 42 | F 50 |
| Incubation period in days : | 49 | 36 | 53 | 57 | 35 | 71 |
| <i>Carbon Balance Sheet.</i> | | | | | | |
| Carbon in solution (start) ... gm. | 4.952 | 4.952 | 4.944 | 4.944 | 4.944 | 4.944 |
| Carbon in H ₂ SO ₄ | 0.012 | 0.028 | 0.009 | nil | 0.019 | 0.002 |
| " in CO ₂ | 2.196 | 2.216 | 2.049 | 1.256 | 1.866 | 2.058 |
| " in mycelium | 0.662 | 0.677 | 0.994 | 0.901 | 0.436 | 1.149 |
| " in solution (end) | 1.944 | 1.933 | 1.784 | 2.693 | 2.494 | 1.602 |
| " accounted for | 4.814 | 4.854 | 4.836 | 4.850 | 4.815 | 4.811 |
| " accounted for ... per cent. | 97.2 | 98.0 | 97.8 | 98.1 | 97.4 | 97.3 |
| <i>Analysis of Solution.</i> | | | | | | |
| Carbon in residual glucose ... gm. | 0.287 | 0.004 | 0.444 | 2.414 | 0.100 | 0.626 |
| " in CO ₂ in solution | 0.001 | 0.009 | 0.017 | 0.016 | 0.010 | nil |
| " in volatile acids | 0.016 | 0.007 | 0.049 | 0.009 | 0.013 | 0.017 |
| " in non-volatile acids | 0.136 | 0.134 | 0.189 | 0.207 | 0.125 | 0.348 |
| " in volatile neutral compounds .. | 0.799 | 1.295 | 0.641 | 0.020 | 1.819 | 0.014 |
| " in synthetic compounds .. | 0.067 | 0.045 | 0.102 | 0.089 | 0.066 | 0.240 |
| Total carbon accounted for | 1.306 | 1.494 | 1.442 | 2.755 | 2.133 | 1.245 |
| " " in solution | 1.944 | 1.933 | 1.784 | 2.693 | 2.494 | 1.602 |
| Carbon unaccounted for (by difference) | 0.638 | 0.439 | 0.342 | Surplus of 0.062 | 0.361 | 0.357 |
| <i>Residual Glucose.</i> | | | | | | |
| Glucose (by polarimeter) ... per cent. | 0.199 | 0.046 | 0.195 | 1.123 | 0.071 | 0.297 |
| " (SHAFFER-HARTMANN) .. | 0.143 | 0.002 | 0.222 | 1.207 | 0.050 | 0.313 |
| " (WOOD-OST) | — | — | — | 1.210 | — | 0.332 |
| " (by alkaline iodine) .. | 0.226 | 0.052 | 0.261 | 1.226 | 0.113 | 0.362 |
| <i>Acids.</i> | | | | | | |
| Titration (N/1 acid) c.c. | Decrease of 0.1 | Decrease of 0.5 | 1.9 | 1.2 | 1.1 | 3.5 |
| Volatile acids (N/1 acid) | 0.78 | 1.59 | 2.44 | 0.32 | 1.40 | 1.09 |
| Barium salts (weight) gm. | 0.066 | 0.115 | 0.252 | 0.027 | 0.136 | 0.066 |
| Calcium salts (weight) | 0.673 | 0.419 | 0.881 | 1.141 | 0.589 | 1.287 |
| Volume of oxygen absorbed ... c.c. | 2910 | 2475 | 2452 | 1698 | 1815 | 3353 |
| Respiration coefficient | 1.41 | 1.68 | 1.57 | 1.40 | 1.93 | 1.14 |
| Mycelium (weight) gm. | 1.295 | 1.387 | 1.659 | 1.495 | 0.901 | 2.342 |
| " (carbon) per cent. | 51.2 | 48.8 | 59.9 | 60.2 | 48.4 | 49.5 |

These two strains agree in the unusually large percentage of carbon in the mycelium, 59·5 per cent. and 60·2 per cent., being very similar in this respect to three strains of *Scopulariopsis* given in Table XIII. which give figures of 62·9 per cent., 62·2 per cent. and 60·3 per cent.

GROUP XI.

Division II.—Asymmetrica.

Section V. Asymmetrica-funiculosa.

Subsection 1. Funiculosa-divaricata.

Two species are included in this group, Ad. 55 (*P. Daleæ* ZALESKI) and Ad. 62 (*P. Godlewskii* ZALESKI). The carbon balance sheets which are given in Table VII are of different types since, while Ad. 55 produces large amounts of "volatile neutral compounds" and has a high respiration coefficient, Ad. 62 produces little if any of this type of compound and has a correspondingly low respiration coefficient. The carbon balance sheet of Ad. 55 is of the same type as those of the strains included in the *P. terrestre* JENSEN series given in Table VIII. It is also of interest to note that in their physiological reactions Ad. 55 and Ad. 62 show considerable differences and are obviously not closely related species from a biochemical point of view. Moreover, another strain of *P. Daleæ* ZALESKI, Ad. 116, for which unfortunately no carbon balance sheet was prepared but which is referred to in Part VII of this series, differs from the strain Ad. 55, since Ad. 116 produces considerable amounts of kojic acid from glucose, while Ad. 55, on the other hand, does not give rise even to traces of this interesting biochemical product.

GROUP XII.

Division II.—Asymmetrica.

Section V. Asymmetrica-funiculosa.

Subsection 2. Funiculosa-typica.

Eight different strains, obtained from various sources, belonging to the large series *P. terrestre* JENSEN, were examined (Ad. 8, Ad. 4, Ad. 5, Ad. 54, Ad. 18, Ad. 50, Ad. 59 and Ad. 9). Their carbon balance sheets are given in Table VIII. These eight strains, with the exception of Ad. 9, represent a well-defined group having the following characteristics:—

- (i) They all have the same type of carbon balance sheet.
- (ii) They all give moderate or negligible amounts of titratable acidity and "carbon in non-volatile acids."
- (iii) They all give large amounts of "volatile neutral compounds" and have correspondingly high respiration coefficients.
- (iv) They all give moderate amounts of "carbon unaccounted for."

TABLE VIII.

| Species of <i>Penicillium</i> : | | <i>P. terrestre</i> JENSEN series. | | | | | | | | | |
|--|--|------------------------------------|-------|-------|--------|--------|--------|-------------|-------|-------|--|
| Catalogue number : | | Ad. 8 | Ad. 4 | Ad. 5 | Ad. 54 | Ad. 18 | Ad. 50 | Ad. 59 | Ad. 9 | | |
| Experiment number : | | F 18 | F 3 | F 4 | F 41 | F 71 | F 38 | F 47 | F 9 | | |
| Incubation period in days : | | 28 | 24 | 35 | 34 | 40 | 49 | 33 | 74 | | |
| <i>Carbon Balance Sheet.</i> | | | | | | | | | | | |
| Carbon in solution (start) ... gm. | | 4-944 | 5-043 | 5-043 | 4-944 | 4-952 | 4-944 | 4-944 | 4-944 | 4-944 | |
| Carbon in H ₂ SO ₄ ... | | 0-013 | 0-017 | 0-020 | 0-021 | 0-012 | 0-024 | 0-028 | 0-001 | 0-001 | |
| " in CO ₂ ... | | 1-562 | 1-626 | 1-924 | 1-994 | 1-930 | 2-257 | 1-988 | 2-282 | 2-282 | |
| " in mycelium ... | | 0-396 | 0-416 | 0-547 | 0-455 | 0-479 | 0-718 | 0-501 | 0-922 | 0-922 | |
| " in solution (end) ... | | 2-865 | 2-848 | 2-405 | 2-393 | 2-412 | 1-767 | 2-321 | 1-621 | 1-621 | |
| " accounted for ... | | 4-836 | 4-907 | 4-896 | 4-863 | 4-833 | 4-766 | 4-838 | 4-826 | 4-826 | |
| " accounted for ... per cent. | | 97-8 | 97-3 | 97-1 | 98-4 | 97-6 | 96-4 | 97-8 | 97-6 | 97-6 | |
| <i>Analysis of Solution.</i> | | | | | | | | | | | |
| Carbon in residual glucose ... gm. | | 0-786 | 0-343 | 0-123 | 0-054 | 0-879 | 0-012 | 0-036 | 0-576 | 0-576 | |
| " in CO ₂ in solution ... | | 0-006 | 0-005 | 0-008 | 0-008 | 0-006 | 0-015 | 0-010 | 0-014 | 0-014 | |
| " in volatile acids ... | | 0-007 | 0-006 | 0-011 | 0-016 | 0-004 | 0-005 | 0-010 | 0-033 | 0-033 | |
| " in non-volatile acids ... | | 0-291 | 0-343 | 0-273 | 0-133 | 0-157 | 0-098 | 0-087 | 0-164 | 0-164 | |
| " in volatile neutral compounds ... | | 1-142 | 1-478 | 1-407 | 1-783 | 0-952 | 1-372 | 1-797 | 0-093 | 0-093 | |
| " in synthetic compounds ... | | 0-200 | 0-053 | 0-133 | 0-189 | 0-065 | 0-076 | 0-075 | 0-066 | 0-066 | |
| Total carbon accounted for ... | | 2-432 | 2-238 | 1-955 | 2-183 | 2-063 | 1-578 | 2-015 | 0-946 | 0-946 | |
| " " in solution ... | | 2-865 | 2-848 | 2-405 | 2-393 | 2-412 | 1-767 | 2-321 | 1-621 | 1-621 | |
| Carbon unaccounted for (by difference) ... | | 0-433 | 0-620 | 0-450 | 0-210 | 0-349 | 0-189 | 0-306 | 0-675 | 0-675 | |
| <i>Residual Glucose.</i> | | | | | | | | | | | |
| Glucose (by polarimeter) ... per cent. | | 0-469 | 0-234 | 0-134 | 0-055 | 0-484 | 0-018 | 0-025 | 0-316 | 0-316 | |
| " (SHAFFER-HAERTMANN) ... | | 0-393 | 0-173 | 0-062 | 0-027 | 0-440 | 0-006 | 0-018 | 0-283 | 0-283 | |
| " (WOOD-Ost) ... | | 0-408 | — | — | — | 0-443 | — | — | — | — | |
| " (by alkaline iodine)... | | 0-443 | 0-231 | 0-124 | 0-097 | 0-516 | 0-043 | 0-082 | 0-317 | 0-317 | |
| <i>Acids.</i> | | | | | | | | | | | |
| Titration (N/1 acid) ... c.c. | | 4-8 | 4-3 | 3-9 | 1-4 | 0-5 | nil | Decrease of | 2-9 | 2-9 | |
| Volatile acids (N/1 acid) ... | | 0-80 | 0-23 | 0-19 | 1-13 | 0-84 | 1-06 | 0-7 | 1-54 | 1-54 | |
| Barium salts (weight) ... gm. | | 0-023 | 0-019 | 0-053 | 0-109 | 0-082 | 0-100 | 0-119 | 0-173 | 0-173 | |
| Calcium salts (weight) ... | | 1-084 | 1-360 | 1-051 | 0-573 | 0-632 | 0-432 | 0-342 | 0-801 | 0-801 | |
| Volume of oxygen absorbed ... c.c. | | 1612 | 1381 | 2002 | 1789 | 2421 | 2598 | 1646 | 3616 | 3616 | |
| Respiration coefficient ... | | 1-82 | 2-20 | 1-80 | 2-08 | 1-49 | 1-63 | 2-26 | 1-19 | 1-19 | |
| Mycelium (weight) ... gm. | | 0-785 | 0-831 | 1-096 | 0-929 | 0-991 | 1-497 | 1-056 | 1-684 | 1-684 | |
| " (carbon) ... per cent. | | 50-4 | 50-1 | 49-9 | 49-0 | 48-3 | 48-0 | 47-5 | 54-8 | 54-8 | |

Ad. 9 seems to be an atypical member of this series in that it only produces negligible amounts of "volatile neutral compounds" and has a low respiration coefficient.

It is of interest to note that all the strains included in this group show closely agreeing physiological characteristics.

Morphological details of Ad. 8, Ad. 9 and Ad. 18 are given in THOM'S '*Penicillia*' on p. 372.

GROUP XIII.

Division II.—Asymmetrica.

Section VI. Fasciculata.

Subsection 1. Sclerotigena.

One strain of *P. gladioli* MACHACEK (Ad. 65) and two different strains of *P. italicum* WEHMER (Ad. 84 and Ad. 85) are included in this group, the carbon balance sheets for which are given in Table IX. The three carbon balance sheets are all of the same type, showing moderate amounts of "carbon in volatile neutral compounds" with medium respiration coefficients, negligible amounts of titratable acidity and moderate amounts of "carbon unaccounted for." The positive characteristic of the cultures included in this group, particularly of Ad. 65, is the high percentage of "carbon in mycelium" (62.3 per cent. in Ad. 65). Colour reactions typical of strains of *P. italicum* WEHMER are described in detail in Part XVIII.

GROUP XIV.

Division II.—Asymmetrica.

Section VI. Fasciculata.

Subsection 3. Viridicata.

Included in this group are three strains belonging to the series *P. viridicatum* WESTLING (Ad. 76, Ad. 77 and Ad. 83), together with *P. psittacinum* THOM (Ad. 22) and *P. verrucosum* DIERCKX (Ad. 15). The carbon balance sheets for these five cultures are given in Table X. Ad. 22 is included in this group since its carbon balance sheet is very similar in type to those of Ad. 76 and Ad. 77 although, in THOM'S morphological classification, it occurs among the *Funiculosa-typica* as subsection 2 of the *Asymmetrica-funiculosa*. In this connection it is of interest to note the following quotation from p. 392 of THOM'S book on the *Penicillia*: "Among bright green species placed elsewhere *P. psittacinum* THOM might from its colour and habit be placed here," i.e., in the *P. viridicatum* series.

These five cultures taken together do not form a group agreeing in biochemical characteristics, as indicated by their carbon balance sheets. The four cultures, Ad. 76, Ad. 77, Ad. 83 and Ad. 22, of which the first three are almost indistinguishable in their physiological reactions and morphological characteristics, have carbon balance

TABLE IX.

| Species of <i>Penicillium</i> : | <i>P. gladioli</i> MACHACEK. | <i>P. italicum</i> WEHMER. | |
|---|---------------------------------|-------------------------------|--------|
| Catalogue number : | Ad. 65 | Ad. 84 | Ad. 85 |
| Experiment number : | F 53 | F 83 | F 84 |
| Incubation period in days : | 64 | 62 | 56 |
| <i>Carbon Balance Sheet.</i> | | | |
| Carbon in solution (start) gm. | 4.944 | 4.952 | 4.952 |
| Carbon in H ₂ SO ₄ " | 0.029 | 0.006 | 0.002 |
| " in CO ₂ " | 2.643 | 2.350 | 1.834 |
| " in mycelium " | 0.868 | 0.715 | 0.623 |
| " in solution (end) " | 1.261 | 1.750 | 2.345 |
| " accounted for " | 4.801 | 4.821 | 4.804 |
| " accounted for per cent. | 97.1 | 97.4 | 97.0 |
| <i>Analysis of Solution.</i> | | | |
| Carbon in residual glucose gm. | 0.018 | 0.600 | 1.477 |
| " in CO ₂ in solution " | 0.010 | 0.007 | 0.005 |
| " in volatile acids " | 0.001 | nil | 0.012 |
| " in non-volatile acids " | 0.167 | 0.169 | 0.048 |
| " in volatile neutral compounds " | 0.562 | 0.631 | 0.392 |
| " in synthetic compounds " | 0.099 | 0.086 | 0.065 |
| Total carbon accounted for " | 0.857 | 1.493 | 1.999 |
| " " in solution " | 1.261 | 1.750 | 2.345 |
| Carbon unaccounted for (by difference) " | 0.404 | 0.257 | 0.346 |
| <i>Residual Glucose.</i> | | | |
| Glucose (by polarimeter) per cent. | 0.028 | 0.335 | 0.744 |
| " (SHAFFER-HARTMANN) " | 0.009 | 0.300 | 0.738 |
| " (WOOD-OST) " | — | 0.302 | 0.758 |
| " (by alkaline iodine) " | 0.071 | 0.347 | 0.825 |
| <i>Acids.</i> | | | |
| Titration (N/1 acid) c.c. | 0.3 | 0.4 | 0.7 |
| Volatile acids (N/1 acid) " | 1.04 | nil | 0.87 |
| Barium salts (weight) gm. | 0.054 | 0.026 | 0.079 |
| Calcium salts (weight) " | 0.721 | 0.842 | 0.226 |
| Volume of oxygen absorbed c.c. | 3818 | 3328 | 2738 |
| Respiration coefficient " | 1.30 | 1.32 | 1.25 |
| Mycelium (weight) gm. | 1.392 | 1.367 | 1.175 |
| " (carbon) per cent. | 62.3 | 52.3 | 53.0 |

TABLE X.

| Species of <i>Penicillium</i> : | <i>P. viridicatum</i> WESTLING. | | <i>P. psittacinum</i> THOM. | | <i>P. verrucosum</i> DIERCKX. | | <i>P. crustosum</i> THOM. | | <i>P. expansum</i> (LINK) THOM series. | | <i>P. Schlegelii</i> BOAS. | | <i>P. cornubiferum</i> WESTLING. | |
|---|---------------------------------|--------|-----------------------------|--------|-------------------------------|--------|---------------------------|--------|--|--------|----------------------------|--------|----------------------------------|--------|
| Catalogue number : | Ad. 76 | Ad. 77 | Ad. 83 | Ad. 22 | Ad. 15 | Ad. 16 | Ad. 1 | Ad. 58 | Ad. 51 | Ad. 61 | Ad. 64 | Ad. 51 | Ad. 61 | Ad. 64 |
| Experiment number : | F 57 | F 58 | F 80 | F 22 | F 70 | F 61 | F 1 | F 46 | F 39 | F 49 | F 52 | F 39 | F 49 | F 52 |
| Incubation period in days : | 36 | 52 | 70 | 49 | 31 | 47 | 37 | 79 | 48 | 41 | 46 | 48 | 41 | 46 |
| <i>Carbon Balance Sheet.</i> | | | | | | | | | | | | | | |
| Carbon in solution (start) ... gm. | 4.944 | 4.944 | 4.952 | 4.944 | 4.952 | 4.944 | 5.043 | 4.944 | 4.944 | 4.944 | 4.944 | 4.944 | 4.944 | 4.944 |
| Carbon in H_2SO_4 ... " | 0.004 | 0.014 | 0.002 | 0.009 | 0.022 | 0.029 | 0.006 | 0.042 | 0.009 | 0.024 | 0.023 | 0.009 | 0.024 | 0.023 |
| " in CO_2 ... " | 1.361 | 2.019 | 1.978 | 1.063 | 1.947 | 2.313 | 1.824 | 2.957 | 2.035 | 2.276 | 2.097 | 2.035 | 2.276 | 2.097 |
| " in mycelium ... " | 0.654 | 0.971 | 1.102 | 0.437 | 0.542 | 0.627 | 0.671 | 0.896 | 0.694 | 0.585 | 0.634 | 0.694 | 0.585 | 0.634 |
| " in solution (end) ... " | 2.796 | 1.769 | 1.694 | 3.342 | 2.335 | 1.840 | 2.354 | 0.904 | 2.102 | 1.931 | 1.956 | 2.102 | 1.931 | 1.956 |
| " accounted for ... " | 4.815 | 4.773 | 4.776 | 4.851 | 4.846 | 4.809 | 4.855 | 4.799 | 4.840 | 4.816 | 4.710 | 4.840 | 4.816 | 4.710 |
| " accounted for ... per cent. | 97.4 | 96.5 | 96.4 | 98.1 | 97.9 | 97.3 | 96.3 | 97.1 | 97.9 | 97.4 | 95.3 | 97.9 | 97.4 | 95.3 |
| <i>Analysis of Solution.</i> | | | | | | | | | | | | | | |
| Carbon in residual glucose ... gm. | 0.868 | 0.088 | 0.067 | 1.428 | 0.070 | 0.006 | 0.599 | 0.008 | 0.134 | 0.040 | 0.032 | 0.134 | 0.040 | 0.032 |
| " in CO_2 in solution ... " | nil | nil | nil | nil | 0.006 | 0.006 | 0.009 | 0.021 | 0.009 | 0.009 | 0.004 | 0.009 | 0.009 | 0.004 |
| " in volatile acids ... " | 0.002 | 0.001 | 0.002 | 0.012 | 0.004 | 0.010 | 0.013 | 0.015 | 0.016 | 0.009 | 0.014 | 0.016 | 0.009 | 0.014 |
| " in non-volatile acids ... " | 0.576 | 0.364 | 0.718 | 0.453 | 0.126 | 0.133 | 0.233 | 0.126 | 0.178 | 0.135 | 0.162 | 0.178 | 0.135 | 0.162 |
| " in volatile neutral compounds ... " | 0.115 | 0.409 | 0.004 | 0.540 | 1.582 | 1.258 | 0.875 | 0.533 | 0.669 | 1.426 | 1.268 | 0.669 | 1.426 | 1.268 |
| " in synthetic compounds ... " | 0.097 | 0.163 | 0.074 | 0.084 | 0.026 | 0.045 | 0.101 | 0.068 | 0.110 | 0.085 | 0.126 | 0.110 | 0.085 | 0.126 |
| Total carbon accounted for ... " | 1.658 | 1.030 | 0.865 | 2.517 | 1.814 | 1.458 | 1.830 | 0.771 | 1.116 | 1.704 | 1.606 | 1.116 | 1.704 | 1.606 |
| " " in solution ... " | 2.796 | 1.769 | 1.694 | 3.342 | 2.335 | 1.840 | 2.354 | 0.904 | 2.102 | 1.931 | 1.956 | 2.102 | 1.931 | 1.956 |
| Carbon unaccounted for (by difference) .. | 1.138 | 0.739 | 0.829 | 0.825 | 0.521 | 0.382 | 0.624 | 0.133 | 0.986 | 0.227 | 0.350 | 0.986 | 0.227 | 0.350 |
| <i>Residual Glucose.</i> | | | | | | | | | | | | | | |
| Glucose (by polarimeter) ... per cent. | 0.635 | 0.088 | 0.239 | 0.879 | 0.075 | 0.041 | 0.390 | nil | 0.099 | 0.025 | 0.028 | 0.099 | 0.025 | 0.028 |
| " (SHAFFER-HARTMANN) ... " | 0.434 | 0.044 | 0.034 | 0.714 | 0.035 | 0.003 | 0.299 | 0.004 | 0.067 | 0.020 | 0.016 | 0.067 | 0.020 | 0.016 |
| " (WOOD-OST) ... " | 0.457 | — | — | 0.716 | — | — | 0.250 | — | — | — | — | — | — | — |
| " (by alkaline iodine) ... " | 0.515 | 0.058 | 0.046 | 0.735 | 0.088 | 0.082 | 0.336 | 0.072 | 0.269 | 0.078 | 0.121 | 0.269 | 0.078 | 0.121 |
| <i>Acids.</i> | | | | | | | | | | | | | | |
| Titration (N/1 acid) ... c.c. | 10.1 | 5.4 | 8.3 | 8.7 | 0.2 | 0.3 | 2.0 | 0.8 | 1.6 | 0.7 | 1.9 | 1.6 | 0.7 | 1.9 |
| Volatile acids (N/1 acid) ... " | 0.37 | 0.35 | 0.06 | 0.33 | 0.83 | 1.45 | 0.54 | 1.47 | 1.52 | 0.73 | 0.57 | 1.52 | 0.73 | 0.57 |
| Barium salts (weight) ... gm. | 0.012 | 0.066 | 0.015 | 0.045 | 0.028 | 0.040 | 0.042 | 0.098 | 0.103 | 0.078 | 0.063 | 0.103 | 0.078 | 0.063 |
| Calcium salts (weight) ... " | 1.691 | 1.159 | 2.410 | 1.554 | 0.452 | 0.482 | 0.995 | 0.637 | 0.802 | 0.616 | 0.540 | 0.802 | 0.616 | 0.540 |
| Volume of oxygen absorbed ... c.c. | 2124 | 2990 | 3486 | 1421 | 1825 | 2638 | 2206 | 4574 | 2630 | 2394 | 2419 | 2630 | 2394 | 2419 |
| Respiration coefficient ... " | 1.19 | 1.26 | 1.06 | 1.40 | 2.00 | 1.64 | 1.55 | 1.22 | 1.45 | 1.78 | 1.62 | 1.45 | 1.78 | 1.62 |
| Mycelium (weight) ... gm. | 1.445 | 2.071 | 2.311 | 0.928 | 1.168 | 1.330 | 1.341 | 1.804 | 1.297 | 1.225 | 1.259 | 1.297 | 1.225 | 1.259 |
| " (carbon) ... per cent. | 45.3 | 46.9 | 47.7 | 47.1 | 46.6 | 47.2 | 50.0 | 49.6 | 53.5 | 47.7 | 50.4 | 53.5 | 47.7 | 50.4 |

sheets showing the following characteristics: they all give large amounts of "carbon unaccounted for," high titratable acidity present entirely in the form of non-volatile acids, but Ad. 76, Ad. 77 and Ad. 22 give moderate amounts of "carbon in volatile neutral compounds" with medium respiration coefficients, while, on the other hand, Ad. 83 shows a complete absence of this type of metabolic product and has a respiration coefficient approximating to unity. Ad. 15, however, has a carbon balance sheet which, while in other respects very similar to those of Ad. 76, Ad. 77 and Ad. 22, is differentiated by the fact that it does not produce any appreciable amount of titratable acidity.

GROUP XV.

Division II.—Asymmetrica.

Section VI. Fasciculata.

Subsection 4. Glauca.

This subsection is divided by THOM into a number of series. The following species, which are arranged in their appropriate series, have been examined and their carbon balance sheets are given in Table X.

Series *Crustaceum*. Ad. 16, *P. crustosum* THOM.

Series *Restrictum*. None.

Series *Expansum*. Ad. 1, Ad. 58, strains of *P. expansum* (LINK) THOM.

Series *Italicum*. Ad. 84, Ad. 85. *P. italicum* WEHMER. The carbon balance sheets for these two strains have already been given in Table IX.

Series ——. Ad. 51, *P. Schneggii* BOAS.

Series *Urticae-patulum*. None.

The species included in this group have carbon balance sheets of similar types, which have no very interesting characteristics except that all the species form considerable amounts of "volatile neutral compounds" and have correspondingly high respiration coefficients. Ad. 51 and, to a smaller extent, Ad. 1 further give rise to considerable amounts of "carbon unaccounted for."

A morphological description of Ad. 16 is given in THOM's '*Penicillia*' on p. 399, and of Ad. 51 on p. 417.

GROUP XVI.

Division II.—Asymmetrica.

Section VI.—Fasciculata.

Subsection 5. Coremiella.

Included in this group are two different strains of *P. corymbiferum* WESTLING (Ad. 61 and Ad. 64). Their carbon balance sheets, which are given in Table X, have only one outstanding feature, namely the very large figure for "carbon in volatile neutral

compounds" and the correspondingly high respiration coefficients. The titratable acidity, the "carbon in non-volatile acids," and the "carbon unaccounted for" are all low. The balance sheets are very similar in type and this biochemical similarity is further substantiated by the similarity in their physiological characteristics.

A morphological description of Ad. 64 is given in THOM'S '*Penicillia*' on p. 424.

GROUP XVII.

Division III.—Biverticillata-symmetrica.

Section I. Ascogena.

Three species are included in this section, Ad. 39, *P. avellaneum* THOM and TURESSON; Ad. 20, *P. Kiliense* WEIDEMANN; and Ad. 101, *P. spiculisporem* LEHMAN.

Section II. Coremigena.

Two different strains of *P. Duclauxi* DELACROIX (Ad. 63 and Ad. 75) are included in this section.

Section III. Luteo-virida.

Subsection IIIa. Funiculosa.

- (i) *Luteo-viride-pinophilum* series. One strain of *P. pinophilum* HEDGCOCK, Ad. 41, is included in this series.
- (ii) *P. funiculosum* series. None.
- (iii) *P. Herquei* series. One strain of *P. Herquei* BAINIER and SARTORY, Ad. 43, is included in this series.

Subsection IIIb. Luteo-purpurogena.

- (i) *P. rugulosum* series. One strain of *P. rugulosum* THOM, Ad. 27, is included in this series.
- (ii) *P. purpurogenum* series. One strain of *P. purpurogenum* STOLL, Ad. 36, is included in this series.
- (iii) *P. luteum* series (non-ascosporic). The non-ascosporic strain of *P. luteum* ZUKAL, Ad. 30, is included in this series.

The carbon balance sheets for all the above species are given in Table XI. They are, however, of such different types that it is impossible to draw any general conclusions as to the biochemical grouping of these species. Thus, while of the three species included in Section I, *Ascogena*, Ad. 39 and Ad. 20 give only small amounts of any metabolic product other than CO₂, Ad. 101 gives the largest amount of "volatile neutral compounds" and has the highest respiration coefficient of any species of *Penicillium* examined. The production by this species of an apparently specific biochemical product, *i.e.*, the lactone of γ -hydroxy- $\beta\delta$ -dicarboxypentadecic acid, is

| Species of <i>Penicillium</i> : | <i>P. avellaneum</i> THOM and TUKESON. | <i>P. Kiliense</i> WEIDE- MANN. | <i>P. spiculi- sporum</i> LEHMAN. | <i>P. Duclauxi</i> DELAEROIX. | | <i>P. pin- ophilum</i> HEDGECOCK. | <i>P. Herquei</i> BAINIER and SARTORY. | <i>P. rugulosum</i> THOM. | <i>P. purpuro- genum</i> STOLL. | <i>P. luteum</i> ZUKAL. |
|--|--|---------------------------------------|--|----------------------------------|----------------|--|---|------------------------------|--|----------------------------|
| Catalogue number: | Ad. 39 | Ad. 20 | Ad. 101 | Ad. 63 | Ad. 75 | Ad. 41 | Ad. 43 | Ad. 27 | Ad. 36 | Ad. 30 |
| Experiment number: | F 26 | F 116 | F 88 | F 51 | F 56 | F 29 | F 31 | F 10 | F 24 | F 12 |
| Incubation period in days: | 81 | 70 | 24 | 21 | 42 | 82 | 69 | 55 | 35 | 68 |
| <i>Carbon Balance Sheet.</i> | | | | | | | | | | |
| Carbon in solution (start) ... gm. | 4.944 | 4.901 | 4.807 | 4.944 | 4.944 | 4.944 | 4.944 | 4.944 | 4.944 | 4.944 |
| Carbon in H_2SO_4 ... " | 0.001 | nil | 0.022 | 0.019 | 0.036 | 0.001 | 0.005 | 0.001 | 0.024 | 0.001 |
| " in CO_2 ... " | 2.555 | 1.610 | 1.638 | 1.739 | 2.318 | 1.807 | 2.129 | 1.935 | 2.042 | 2.017 |
| " in mycelium ... " | 0.605 | 0.807 | 0.181 | 0.365 | 0.460 | 0.546 | 0.655 | 0.686 | 0.620 | 1.252 |
| " in solution (end) ... " | 1.717 | 2.278 | 2.757 | 2.719 | 2.009 | 2.516 | 2.114 | 2.249 | 2.173 | 1.505 |
| " accounted for ... " | 4.878 | 4.695 | 4.598 | 4.842 | 4.823 | 4.870 | 4.903 | 4.871 | 4.859 | 4.775 |
| " accounted for ... per cent. | 98.7 | 95.8 | 95.7 | 97.9 | 97.5 | 98.5 | 99.2 | 98.5 | 98.3 | 96.6 |
| <i>Analysis of Solution.</i> | | | | | | | | | | |
| Carbon in residual glucose ... gm. | 1.268 | 1.781 | 0.134 | 0.054 | 0.028 | 2.038 | 0.274 | 1.488 | 0.022 | 0.774 |
| " in CO_2 in solution ... " | 0.011 | 0.006 | 0.020 | 0.013 | 0.015 | 0.004 | 0.005 | 0.007 | nil | 0.007 |
| " in volatile acids ... " | 0.028 | 0.008 | nil | 0.037 | nil | 0.038 | 0.001 | nil | 0.002 | 0.001 |
| " in non-volatile acids ... " | 0.206 | 0.146 | 0.094 | 0.100 | 0.132 | 0.288 | 0.336 | 0.359 | 0.220 | 0.477 |
| " in volatile neutral compounds ... " | 0.015 | 0.006 | 2.381 | 1.916 | 1.621 | 0.017 | 0.232 | 0.008 | 1.423 | 0.002 |
| " in synthetic compounds ... " | 0.090 | 0.226 | 0.003 | 0.040 | 0.084 | 0.088 | 0.050 | 0.048 | 0.188 | 0.265 |
| Total carbon accounted for ... " | 1.618 | 2.173 | 2.632 | 2.560 | 1.880 | 2.473 | 0.898 | 1.910 | 1.855 | 1.526 |
| " " in solution ... " | 1.717 | 2.278 | 2.757 | 2.719 | 2.009 | 2.516 | 2.114 | 2.249 | 2.173 | 1.505 |
| Carbon unaccounted for (by difference) ... " | 0.099 | 0.105 | 0.125 | 0.159 | 0.129 | 0.043 | 1.216 | 0.339 | 0.318 | Surplus of 0.021 |
| <i>Residual Glucose.</i> | | | | | | | | | | |
| Glucose (by polarimeter) ... per cent. | 0.588 | 0.924 | 0.060 | 0.217 | nil | 1.061 | 0.173 | 0.738 | 0.063 | 0.275 |
| " (SHAFFER-HARTMANN) ... " | 0.634 | 0.890 | — | 0.227 | 0.014 | 1.019 | 0.137 | 0.744 | 0.011 | 0.387 |
| " (Wood-Ost) ... " | 0.634 | 0.924 | 0.067 | — | — | 1.040 | — | 0.788 | — | 0.270 |
| " (by alkaline iodine) ... " | 0.711 | 0.915 | 0.146 | 0.268 | 0.122 | 1.017 | 0.172 | 0.791 | 0.048 | 0.454 |
| <i>Acids.</i> | | | | | | | | | | |
| Titration (N/1 acid) ... c.c. | 1.1 | 0.6 | 1.5 | 1.4 | Decrease of | 3.0 | 5.2 | 1.4 | 2.0 | 0.6 |
| Volatile acids (N/1 acid) ... " | 1.10 | 0.77 | nil | 2.66 | 0.56 | 2.04 | nil | nil | 0.59 | 0.42 |
| Barium salts (weight) ... gm. | 0.133 | 0.045 | 0.064 | 0.262 | 0.013 | 0.205 | 0.009 | 0.011 | 0.038 | — |
| Calcium salts (weight) ... " | 0.781 | 1.260 | 0.398 | 0.568 | 0.581 | 1.106 | — | 0.740 | 0.866 | 1.918 |
| Volume of oxygen absorbed ... c.c. | 4279 | 2881 | 769 | 1157 | 2435 | 3065 | 3415 | 3255 | 2151 | 3269 |
| Respiration coefficient ... " | 1.12 | 1.05 | 4.03 | 2.82 | 1.79 | 1.10 | 1.17 | 1.11 | 1.77 | 1.04 |
| Mycelium (weight) ... gm. | 1.152 | 1.555 | 0.392 | 0.748 | 0.886 | 0.990 | 1.335 | 1.342 | 1.212 | 2.397 |
| " (carbon) ... per cent. | 52.2 | 51.9 | 46.2 | 48.8 | 51.9 | 55.1 | 49.1 | 51.1 | 51.2 | 52.2 |

dealt with in Part XVI. This difference in biochemical activities is readily explicable when it is remembered that the grouping of these species is admitted by THOM to be largely an arbitrary one. Thus, on p. 445 of his book, THOM says, "In making this aggregate, *P. avellaneum* THOM and TURESSON, and *P. spiculisporem* LEHMAN, whose affinities are doubtful, have been included with a truly homogeneous series including *P. luteum* and its allies."

In Section II, *Coremigena*, it is interesting to note that the two different strains of *P. Duclauxi*, Ad. 63 and Ad. 75, give balance sheets which are very similar in type, having as their only outstanding characteristic the production of large amounts of "volatile neutral compounds" and correspondingly high respiration coefficients.

The two species included in subsection IIIa, *P. pinophilum*, Ad. 41, and *P. Herquei*, Ad. 43, have also carbon balance sheets of different types, since, while Ad. 43 gives a very large figure for "carbon unaccounted for," Ad. 41 produces only negligible amounts of products of this type. In other respects the two carbon balance sheets are not very dissimilar.

The three species included in subsection IIIb, *P. rugulosum*, Ad. 27, *P. purpurogenum*, Ad. 36, and *P. luteum*, Ad. 30, also have different types of carbon balance sheets. Thus, while Ad. 27 and Ad. 30 produce practically no "volatile neutral compounds" and have respiration coefficients approaching unity, Ad. 36 gives a large yield of this type of product and has a very high respiration coefficient.

The carbon balance sheet for *P. luteum* (non-ascosporic form), Ad. 30, has several interesting characteristics. Of outstanding interest is the fact that while the titratable acidity is only 0.6 c.c., the "carbon in non-volatile acids" gives the relatively high figure of 0.477 gm., corresponding to about 11 per cent. of some compound precipitated as a calcium salt in 80 per cent. alcohol. The explanation for this will be found in Part XIII, in which are described the preparation and properties of a mucilaginous material which is a complex built up of units of a malonyl polyglucose. It titrates as an acid having a combining weight of between 400 and 500, and is precipitable as a calcium salt from 80 per cent. alcohol. The same types of figures are also given by *P. rugulosum*, Ad. 27, in which the titratable acidity is 1.4 c.c. and the "carbon in non-volatile acids" 0.359 gm, and by *P. tardum*, Ad. 45 (see Table XII), in which the figures are 0.6 c.c. and 0.392 gm. respectively. The nature of the products responsible for these figures in the case of Ad. 27 and Ad. 45 has not yet been investigated.

GROUP XVIII.

Division III. *Biverticillata-symmetrica*.

Section IV. *Miscellanea*.

Two strains of *P. tardum* THOM, Ad. 45 and Ad. 47, are included in this group. Their carbon balance sheets, which are given in Table XII, are so different in type as to lead to the belief that, from a biochemical point of view, these two strains are widely different.

TABLE XII.

| Species of <i>Penicillium</i> : | | | | | | <i>P. tardum</i> THOM. | |
|--|-----|-----|-----|-----|-----------|------------------------|--------|
| Catalogue number : | | | | | | Ad. 45 | Ad. 47 |
| Experiment number : | | | | | | F 33 | F 35 |
| Incubation period in days : | | | | | | 70 | 45 |
| <i>Carbon Balance Sheet.</i> | | | | | | | |
| Carbon in solution (start) | ... | ... | ... | ... | gm. | 4.944 | 4.944 |
| Carbon in H ₂ SO ₄ | ... | ... | ... | ... | " | nil | 0.015 |
| " in CO ₂ ... | ... | ... | ... | ... | " | 2.107 | 2.037 |
| " in mycelium | ... | ... | ... | ... | " | 0.940 | 0.449 |
| " in solution (end) | ... | ... | ... | ... | " | 1.743 | 2.352 |
| " accounted for | ... | ... | ... | ... | " | 4.790 | 4.853 |
| " accounted for | ... | ... | ... | ... | per cent. | 96.9 | 98.2 |
| <i>Analysis of Solution.</i> | | | | | | | |
| Carbon in residual glucose | ... | ... | ... | ... | gm. | 1.090 | 0.576 |
| " in CO ₂ in solution | ... | ... | ... | ... | " | 0.009 | 0.008 |
| " in volatile acids | ... | ... | ... | ... | " | 0.005 | 0.026 |
| " in non-volatile acids... | ... | ... | ... | ... | " | 0.392 | 0.102 |
| " in volatile neutral compounds | ... | ... | ... | ... | " | 0.002 | 1.065 |
| " in synthetic compounds | ... | ... | ... | ... | " | 0.058 | 0.045 |
| Total carbon accounted for | ... | ... | ... | ... | " | 1.556 | 1.822 |
| " in solution | ... | ... | ... | ... | " | 1.743 | 2.352 |
| Carbon unaccounted for (by difference) | ... | ... | ... | ... | " | 0.187 | 0.530 |
| <i>Residual Glucose.</i> | | | | | | | |
| Glucose (by polarimeter) | ... | ... | ... | ... | per cent. | 0.482 | 0.276 |
| " (SHAFFER-HARTMANN) | ... | ... | ... | ... | " | 0.545 | 0.288 |
| " (WOOD-OST) | ... | ... | ... | ... | " | 0.544 | — |
| " (by alkaline iodine) | ... | ... | ... | ... | " | 0.562 | 0.402 |
| <i>Acids.</i> | | | | | | | |
| Titration (N/1 acid) | ... | ... | ... | ... | c.c. | 0.6 | 1.2 |
| Volatile acids (N/1 acid) | ... | ... | ... | ... | " | 0.01 | 1.59 |
| Barium salts (weight) | ... | ... | ... | ... | gm. | 0.004 | 0.157 |
| Calcium salts (weight) | ... | ... | ... | ... | " | 1.353 | 0.584 |
| Volume of oxygen absorbed | ... | ... | ... | ... | c.c. | 3655 | 2440 |
| Respiration coefficient | ... | ... | ... | ... | ... | 1.05 | 1.56 |
| Mycelium (weight) | ... | ... | ... | ... | gm. | 1.893 | 0.916 |
| " (carbon) | ... | ... | ... | ... | per cent. | 49.7 | 49.0 |

GROUP XIX.

Genus *Gliocladium* CORDA.Genus *Scopulariopsis* BAINIER.Genus *Pæcilomyces* BAINIER.Unnamed species of *Penicillium*.

Included in this group are seven species which are grouped together more for the sake of convenience than for any morphological or biochemical relationships. In doing this, the arrangement adopted by THOM in his book has been followed. The species included are :—

- (i) Ad. 38—belonging to the genus *Gliocladium* and probably a strain of *Gliocladium roseum* BAINIER.
- (ii) Ad. 40, Ad. 70 and Ad. 72—three different strains of the species formerly known as *P. brevicaulis* SACCARDO, but now transferred to the genus *Scopulariopsis* and hence becoming strains of *Scopulariopsis brevicaulis* (SACCARDO) BAINIER.
- (iii) Ad. 44—formerly known as *P. divaricatum* THOM and now transferred to the genus *Pæcilomyces* and becoming a strain of *Pæcilomyces varioti* BAINIER.
- (iv) Ad. 3 and Ad. 57—two very similar unnamed species of *Penicillium*, the history of which is given in the Appendix.

The carbon balance sheets for these species are given in Table XIII and the only points of outstanding interest in them are the following :—

- (i) The three strains of *Scopulariopsis* give balance sheets which are all of the same type and indicate that these strains do not produce, in appreciable quantity, any product other than CO₂. In this connection the following quotation from p. 511 of THOM's book is of interest: "The species (*Scopulariopsis*) appear as agents of decomposition after the usual green *Penicillia* have ceased to be active; that is in the later stages of decay processes."
- (ii) The carbon balance sheets of Ad. 3 and Ad. 57 are so similar in type that these two species are obviously closely related biochemically. Both species give rise to large amounts of "volatile neutral compounds," have high respiration coefficients and moderate amounts of "carbon unaccounted for."

Discussion of Results.

The carbon balance sheets submitted for a large number of different species of *Penicillium* show very wide variations in type, exactly as was noticed with species of *Aspergillus*. The species and their carbon balance sheets have been arranged in the same order as is followed in THOM's '*Penicillia*,' but the close agreement between

TABLE XIII.

| | <i>Gliocladium roseum</i> BAINIER. | <i>Scopulariopsis brevicaulis</i> (SACCARDO) BAINIER. | | | <i>Pæcilomyces varioti</i> BAINIER. | <i>Penicillium</i> species unnamed. | |
|---|---------------------------------------|--|-----------------------|--------|--|--|--------|
| Catalogue number : | Ad. 38 | Ad. 40 | Ad. 70 | Ad. 72 | Ad. 44 | Ad. 3 | Ad. 57 |
| Experiment number : | F 28 | F 27 | F 77 | F 81 | F 32 | F 2 | F 45 |
| Incubation period in days : | 63 | 49 | 60 | 63 | 63 | 39 | 59 |
| <i>Carbon Balance Sheet.</i> | | | | | | | |
| Carbon in solution (start) gm. | 4.944 | 4.944 | 4.952 | 4.952 | 4.944 | 5.043 | 4.944 |
| Carbon in H ₂ SO ₄ " | 0.013 | 0.001 | 0.001 | nil | 0.022 | 0.014 | 0.028 |
| " in CO ₂ " | 2.109 | 2.099 | 1.913 | 1.824 | 2.557 | 2.012 | 1.954 |
| " in mycelium " | 0.565 | 1.284 | 1.361 | 1.113 | 0.862 | 0.724 | 0.509 |
| " in solution (end)... .. " | 2.160 | 1.522 | 1.595 | 1.879 | 1.395 | 2.088 | 2.335 |
| " accounted for " | 4.847 | 4.906 | 4.870 | 4.816 | 4.836 | 4.838 | 4.826 |
| " accounted for per cent. | 98.0 | 99.2 | 98.3 | 97.5 | 97.8 | 95.9 | 97.6 |
| <i>Analysis of Solution.</i> | | | | | | | |
| Carbon in residual glucose gm. | 0.628 | 1.054 | 1.022 | 1.490 | 0.014 | 0.064 | 0.346 |
| " in CO ₂ in solution " | 0.007 | 0.022 | 0.004 | 0.008 | 0.003 | 0.007 | 0.002 |
| " in volatile acids " | 0.063 | 0.033 | 0.025 | 0.036 | nil | 0.006 | 0.005 |
| " in non-volatile acids " | 0.186 | 0.122 | 0.118 | 0.113 | 0.198 | 0.253 | 0.195 |
| " in volatile neutral compounds " | 0.893 | 0.007 | 0.004 | nil | 0.555 | 1.074 | 1.168 |
| " in synthetic compounds " | 0.017 | 0.074 | 0.034 | 0.028 | 0.118 | 0.179 | 0.083 |
| Total carbon accounted for " | 1.794 | 1.312 | 1.207 | 1.675 | 0.888 | 1.583 | 1.799 |
| " " in solution " | 2.160 | 1.522 | 1.595 | 1.879 | 1.395 | 2.088 | 2.335 |
| Carbon unaccounted for (by difference) .. | 0.366 | 0.210 | 0.388 | 0.204 | 0.507 | 0.505 | 0.536 |
| <i>Residual Glucose.</i> | | | | | | | |
| Glucose (by polarimeter) per cent. | 0.336 | 0.527 | 0.521 | 0.678 | 0.050 | 0.101 | 0.257 |
| " (SHAFFER-HARTMANN) " | 0.314 | 0.527 | 0.511 | 0.745 | 0.007 | 0.032 | 0.173 |
| " (WOOD-OST) " | 0.310 | 0.533 | 0.464 | 0.742 | — | — | — |
| " (by alkaline iodine) " | 0.393 | 0.571 | 0.595 | 0.793 | 0.083 | 0.090 | 0.223 |
| <i>Acids.</i> | | | | | | | |
| Titration (N/1 acid) c.c. | 1.9 | Decrease of 0.2 | Decrease of 0.3 | 0.2 | 2.6 | 1.9 | 1.0 |
| Volatile acids (N/1 acid) " | 1.62 | 2.25 | 1.46 | 1.46 | 0.15 | nil | 1.30 |
| Barium salts (weight) gm. | 0.314 | 0.158 | 0.103 | 0.185 | 0.062 | 0.047 | 0.075 |
| Calcium salts (weight) " | 0.723 | 0.541 | 0.410 | 0.511 | 0.850 | 0.855 | 0.778 |
| Volume of oxygen absorbed c.c. | 2753 | 2973 | 2712 | 2853 | 3651 | 2379 | 2189 |
| Respiration coefficient " | 1.44 | 1.33 | 1.27 | 1.20 | 1.37 | 1.58 | 1.67 |
| Mycelium (weight) gm. | 1.056 | 2.040 | 2.190 | 1.845 | 1.562 | 1.469 | 1.034 |
| " (carbon) per cent. | 53.5 | 62.9 | 62.2 | 60.3 | 55.2 | 49.3 | 49.3 |

the morphological classification and the biochemical classification that was found with species of *Aspergillus*, is not so apparent with species of *Penicillium*, although the majority of the groups of *Penicillium* do agree morphologically and biochemically. In some cases, however, wide divergences have been found where species grouped together morphologically give carbon balance sheets of entirely different types and are obviously not very closely related biochemically. In these cases it is possible that subsequent alteration in the morphological classification will bring our findings more into line with THOM's classification since he himself admits that some species of *Penicillium* are grouped together in his book on somewhat arbitrary lines. We have, indeed, found that certain strains of *Penicillium*, admittedly of the same species, may give rise to large amounts of alcohol, while other strains of the same species produce none of this metabolic product. An example of this is given in Group X, where of two strains of *P. lilacinum* THOM, obtained from different parts of the world, one strain gives 12 per cent. of alcohol and the other strain gives scarcely any. In other respects their carbon balance sheets are very similar. Another instance is given in Part III for different strains of *A. nidulans* EIDAM. We have at present no explanation to offer for what is undoubtedly a very definite biochemical fact, since it must be remembered that in all of these experiments identical cultural conditions were followed. On the other hand there are several instances in which different strains of the same species of *Penicillium*, obtained from different parts of the world, give carbon balance sheets which are almost indistinguishable from one another. Notable examples of these are to be found in Table II for five different strains of *P. (Citromyces) glabrum* WEHMER, and in Table XIII for three different strains of *Scopulariopsis brevicaulis* (SACC.) BAINIER.

It may be well to summarize the results given in this paper by dealing in turn with the outstanding items in the carbon balance sheets.

I. *Growth*.—It may be said that in general the majority of the species of *Penicillium* investigated grow reasonably well on the medium used and under the cultural conditions followed. An outstanding exception is *P. digitatum* SACCARDO, which only begins to show signs of germination on this medium after two to three weeks' incubation but, having once germinated, growth is ultimately quite good. This was found to be the case for three different strains of this species and confirms the observation, previously made by THOM, that *P. digitatum* can only use sodium nitrate as a source of nitrogen with considerable difficulty.

II. *Carbon in Sulphuric Acid Bubbler*.—The class of carbon compounds included under this heading consists of any very volatile organic substance which is absorbed by concentrated sulphuric acid. It is a well-known fact that many species of *Penicillium* under certain cultural conditions have very marked odours, but, under the cultural conditions followed in this paper, only one species of *Penicillium*, *P. digitatum*, gives rise to any marked amount of bodies of this type. The substance responsible has been identified as ethyl acetate and has been shown to be produced by each of the three

different strains of *P. digitatum* investigated. The results obtained are described in detail in Part XVIII.

III. *Carbon in Volatile Acids*.—Consideration of the figures obtained for all species for “carbon in volatile acids” enables one to make an important biochemical generalization. In no case did any species of *Penicillium* produce more than small yields of volatile acids and in many cases this type of body was entirely absent. In fact, the maximum yield from any species of *Penicillium* is that given by *P. lilacinum*, Ad. 32 = 0.049 gm. (Table VII), corresponding to a yield of approximately 1 per cent. A similar absence of the production of volatile acids has already been noticed with species of *Aspergillus* (Part III) and hence this biochemical characteristic of these two great genera of the lower fungi is in very marked contrast to the well-known biochemical characteristic of many of the bacteria of producing large yields of this class of bodies.

IV. *Carbon in Non-volatile Acids*.—All our results go to show that, as with the *Aspergilli* so with the *Penicillia*, the main acids formed are of the non-volatile type, giving calcium salts which are insoluble in 80 per cent. alcohol, the acids themselves being usually polybasic and often hydroxy-acids. The following outstanding examples may be noticed. All the species included in the *P. (Citromyces) Pfefferianum* series (Table I) produce considerable, and in some cases very large, amounts of citric acid, strains Ad. 74, Ad. 80 and Ad. 79 being particularly marked in this respect and giving yields at least as high as any species of *Aspergillus*. Certain other species, particularly those belonging to the *P. chrysogenum* series (Table V), give large yields of gluconic acid, an observation which has been reported in detail in Part XVII for *P. chrysogenum*, Ad. 11. Presumptive evidence of the production of gluconic acid by Ad. 56, Ad. 14, Ad. 35 and Ad. 53, belonging to the same series, is also furnished in Table V by the large differences between glucose as estimated by the polarimeter and by the SHAFFER-HARTMANN method.

V. *Carbon in Volatile Neutral Compounds and Respiration Coefficients*.—The results presented give perfectly definite evidence for the view previously expressed in Part III that even under conditions of restricted aeration many species of *Penicillium* and *Aspergillus* do not produce alcohol, since, while certain large groups, e.g., the *P. terrestre* JENSEN series given in Table VIII, give yields of alcohol approximating to 40 per cent. and have respiration coefficients as high as 2.26, other groups, e.g., strains of the cheese moulds, *P. caseicolum* and *P. camemberti* (Table VI), all the strains of *P. chrysogenum* (Table V) and many others give yields of alcohol which never exceed 0.2–0.3 per cent. and have respiration coefficients approximating to unity.

VI. *Carbon Unaccounted for*.—Several species of *Penicillium* give large yields of “carbon unaccounted for.” The group which is outstanding in this respect is Group V, i.e., the *P. chrysogenum* series. Of six strains included in this group, five give yields of “carbon unaccounted for” varying between 20 and 30 per cent. of the glucose metabolized. The metabolic products of one of these strains, Ad. 11, are described in Part XVII. The *P. viridicatum* series given in Table X, together with *P. puberulum*,

Table V, and *P. Schnegglii*, Table X, give yields approximating to 20 per cent., many other species giving somewhat smaller yields. Obviously any of these species would repay investigation from the point of view of obtaining good yields of some metabolic product which may or may not, of course, be new. On the other hand, there are groups, represented particularly by the cheese moulds, *P. caseicolum* and *P. camemberti* (Table VI), and strains of *Scopulariopsis* (Table XIII) which do not produce from glucose in appreciable amounts any metabolic product other than carbon dioxide and hence, from this point of view, may be disregarded for future work.

VII. *Carbon in Mycelium*.—The majority of the species of *Penicillium* examined have a carbon content in their mycelium of approximately 50 per cent. There are, however, some exceptions to this rule and it is interesting to note that these fall into certain groups. Thus, of two strains of *P. lilacinum* THOM examined (Table VII), the mycelium of one strain, Ad. 32, contains 59·5 per cent. of carbon, while that of the other strain, Ad. 37, contains 60·2 per cent.; and the three strains of *Scopulariopsis* (Table XIII) have carbon contents of 62·9 per cent., 62·2 per cent. and 60·3 per cent. respectively. These figures are quite definite and unmistakable, though their significance is somewhat obscure.

Summary.

A quantitative examination has been made, by the carbon balance sheet method described in Part II, of the types of products formed from glucose by a large number of different species of *Penicillium*. These carbon balance sheets are collected in a number of groups arranged according to the morphological classification of species of *Penicillium* followed in THOM'S book 'The *Penicillia*.' Many of these groups have their own peculiar biochemical characteristics, though the agreement between the morphological classification and the biochemical classification is not so close as was found to be the case with species of *Aspergillus*.

By means of the carbon balance sheets a choice has been made of species suitable for intensive examination with a view to isolating and identifying their metabolic products. The genus *Penicillium* seems to be unusually rich in this type of organism.

APPENDIX.

HISTORY OF SPECIES USED AND DR. THOM'S DIAGNOSES OF SPECIES.

(Note that Dr. THOM's remarks are in each case inserted in inverted commas.)

*I. Monoverticillata.**Section I. Monoverticillata-stricta.**Subsection 1. Sclerotigena.*

None.

Subsection 2. Stricta-floccosa.

- (i) Ad. 74, Ad. 79, Ad. 80. These three species were isolated at Ardeer, Ad. 74 from a distilled-water container, Ad. 79 and Ad. 80 from different samples of mouldy dextrin solution. "Ad. 74 and Ad. 80 in our cultures are practically identical, with a slight difference in appearance in Ad. 79. I have put them together and placed them in the group with *P. spinulosum* or, in deference to the belief that Dr. WEHMER's *Citromyces Pfefferianus* belonged somewhere in this group, *P. Pfefferianum* series. Our notes indicate that they approach *P. viridi-dorsum* BIOURGE, which, in our cultures from the transfer he furnished us, belongs here. These three species are much more characteristic of the *P. Pfefferianum* series than Ad. 71 and Ad. 73 (*q.v.*)."
- (ii) Ad. 78. Isolated at Ardeer from mouldy dextrin paste. "My notes add Ad. 78 to the Ad. 74, Ad. 79, Ad. 80 group."
- (iii) Ad. 21. Isolated at Ardeer from a contamination of a solution of bromthymol blue indicator. "I have put this species with a lot more into the *P. Pfefferianum* group, with WEHMER's *Citromyces* = *P. Pfefferianum*, *P. spinulosum* THOM and a number of BIOURGE's species, in which I based the allocation on the cultures he sent me, which do differ in detail but have too much in common to be differentiated by his description or mine."
- (iv) Ad. 29. Purchased from the British National Collection of Type Cultures, Catalogue No. 591, THOM and CHURCH, Washington 45. "Correctly labelled as *P. spinulosum* but see comments on Ad. 21."
- (v) Ad. 71. Purchased from PRIBRAM of Vienna in 1926. It bore the label on receipt of *Citromyces lacticus* MAZÉ and PERRIER. "Ad. 71 and Ad. 73 belong to the series in which the failure to produce colour is a prominent character and hence I regard these species as being in line with the *P. (Citromyces) Pfefferianum* group."
- (vi) Ad. 73. Purchased from PRIBRAM of Vienna in 1926. It bore the label on receipt of *Citromyces Pfefferianus* WEHMER. "See remarks on Ad. 71."

- (vii) Ad. 115. Purchased from the Centraalbureau at Baarn in 1929, labelled *P. frequentans* WESTLING. No carbon balance sheet was prepared for this species. "My notes place this culture in the *P. spinulosum* or *P. Pfefferianum* series instead of the *P. (Citromyces) glaber* series where my own culture as received from WESTLING many years ago was placed."

Subsection 3. Stricta funiculosa.

None.

Subsection 4. Velutina.

- (i) Ad. 48. Purchased from Baarn in 1925, labelled *P. majusculum* WESTLING. "I find my transfers belong in series with *P. aurantio-violaceum* BOURGE."
- (ii) Ad. 6. Purchased in 1922 from the British National Collection of Type Cultures, labelled *Citromyces* WEHMER species. Ad. 7. Isolated at Ardeer from an agaric. Ad. 67. Purchased in 1926 from the British National Collection of Type Cultures, labelled *Citromyces* B. WEHMER, Catalogue No. 606. Ad. 68. Purchased from the Centraalbureau at Baarn in 1926, labelled *Citromyces glaber*. Ad. 69. Purchased from the Centraalbureau at Baarn, labelled *Citromyces Pfefferianus*. "Ad. 6 is a monoverticillate, velvety strain with spore chains massed into a column. I place it in the composite series assigned as *P. (Citromyces) glabrum* WEHMER series, in which *P. frequentans* WESTLING and several other forms are also placed. There may be considerable quantitative differences among these forms which shade into each other, both in structure and in reactions produced. Ad. 7 is also in the *P. glabrum* series. Cf. Ad. 6. Ad. 67, Ad. 68 and Ad. 69 also belong to the *P. glabrum* group, as I am using it, or to the *Citromyces glaber* group if you prefer to maintain WEHMER's generic name. There seems to be enough cumulative information available to justify the belief that *Citromyces glaber* falls somewhere in this lot of species and *Citromyces Pfefferianus* in the other lot typified by THOM's *P. spinulosum*, as a form known and hence tangible."

Section II. Monoverticillata-Ramigena.

None.

II. *Asymmetrica.*

Section I. Velutina.

Subsection 1. Velutina-elliptica-magna.

- Ad. 52. Purchased from the Centraalbureau at Baarn in 1925, labelled *P. olivaceum* WEHMER. Ad. 81. Isolated at Ardeer in 1926 from a mouldy orange. Ad. 102. Purchased in 1927 from the American Type Culture Collection, Catalogue No. 1113, labelled *P. digitatum*. "Ad. 52, *P. olivaceum*, is correctly named as the olive-coloured rot of oranges, but the name should be changed to

P. digitatum to comply with the rules. Ad. 81 and Ad. 102, each labelled *P. digitatum*, are satisfactory."

Subsection 2. Velutina-divaricata.

- (i) Ad. 42. Purchased from Baarn in 1925, labelled *P. intricatum*. "Ad. 42, labelled *P. intricatum*: our colonies and your notes are closer to *P. Steckii* ZALESKI than to our descriptions of *P. intricatum*, whose original was lost many years ago from our collection, or to any of the forms we have recently allocated to the series with *P. intricatum*."
- (ii) Ad. 23. Received from Mr. F. T. BROOKS of Cambridge, labelled *P. citrinum* THOM. Ad. 95. Purchased in 1927 from the American Collection of Type Cultures, labelled *P. citrinum* THOM, Catalogue No. 1109. Ad. 114. Received from Mr. GALLOWAY, mycologist at the Shirley Institute of the Cotton Research Association, in 1928. It was isolated by him from mildewed cotton and labelled by him *P. aurifluum*. Ad. 23 is the only one of these three species for which a carbon balance sheet has been prepared, but each of the three species is dealt with in Part XIV on the preparation of citrinin. "Ad. 23 varies somewhat in structure from the usual forms of *P. citrinum*, while harmonizing in general reactions with that species, hence we may accept it as *P. citrinum* THOM or some strain near it. Ad. 95 is correctly named as *P. citrinum* THOM. Ad. 114, labelled *P. aurifluum* BIOURGE; in my discussion of BIOURGE's description of this species I express the opinion that he had my type culture of *P. citrinum* and refused to believe it, because he accepted certain notes of WESTLING as having more weight in determining what I had than my own description. You are right—it is *P. citrinum*."

Subsection 3. Radiata (P. chrysogenum series).

- (i) Ad. 11. Isolated at Ardeer from mouldy tobacco. Ad. 24. Received from Mr. F. T. BROOKS of Cambridge. Ad. 56. Purchased from Baarn in 1925, labelled *P. citrinum*. "Ad. 11 is *P. chrysogenum* or at least one of the strains in this composite, which may perhaps be called, as BIOURGE calls his whole series, *Radiata* rather than trying to fix a species binomial to one of them. Ad. 24. My cultures of this species indicate one of the *Radiata*, with colours in reverse and substratum which would carry it away some distance from *P. chrysogenum*, however. Ad. 56, labelled *P. citrinum*, is certainly not *P. citrinum* as I understand it in my transfers of your culture, but one of the *Radiata*, i.e., the *P. chrysogenum*-*P. notatum* series."
- (ii) Ad. 14. Isolated at Ardeer from 3 per cent. sodium azide solution. "Ad. 14 is another of the *Radiata* of BIOURGE. It is perhaps not far from *P. baculatum* WESTLING, but identity to strain is not claimed but rather the use of the name for a number of strains showing the same general variations within the *Radiata*."

- (iii) Ad. 35. Purchased in 1925 from Baarn. "Ad. 35 is *P. notatum* WESTLING. This is a member of the *P. chrysogenum* series with smaller conidia than *P. chrysogenum* itself."
- (iv) Ad. 53. Purchased from Baarn in 1925, labelled *P. Lagerheimi*. "Ad. 53, labelled *P. Lagerheimi* WESTLING: I was never well satisfied by *P. Lagerheimi*. WESTLING sent this to WESTERDIJK about the same time that he sent it to me, and I have notes as to the organism I received, which do not satisfy this nor does the description. I am inclined to adhere to my previous statement that this species is one of the *Radiata* and is nearer to *P. meleagrinum* than to the name given on the tube."

Subsection 4. Velutina-restricta.

Ad. 31. Purchased in 1925 from Baarn, labelled *P. puberulum*. "Ad. 31, labelled *P. puberulum*, with the notation ALSBERG and BLACK strain is correctly named."

Subsection 5. Stellata of BIOURGE.

None.

Subsection 6. Velutina-asperula.

None.

Section II. Brevi-compacta.

Ad. 87. Isolated at Ardeer as a contaminant of a culture of an alga in CZAPEK-DON solution. "Ad. 87 appears to be a member of BIOURGE'S *Hemizonata* or *P. brevi-compactum* series and is near to *P. Biourgeianum* ZALESKI."

Section III. Lanata-typica.

- (i) Ad. 25. Purchased from the British National Collection of Type Cultures, Catalogue No. 602, labelled *P. candidum* LINK. Ad. 49. Purchased from Baarn, labelled *P. Camemberti*, var. *Rogeri* THOM. "Ad. 25 is *P. caseicolum* BAINIER, which is synonymous with *P. Camemberti* var. *Rogeri* of THOM 1911, and with *P. candidum* of MAZÉ and ROGER in the French cheese literature. Ad. 49, labelled *P. Camemberti* var. *Rogeri* THOM = *P. caseicolum* (see Ad. 25) is correctly named." Ad. 34 purchased from Baarn in 1925, labelled *P. Camemberti*. "Ad. 34 is correctly named as *P. Camemberti* THOM."
- (ii) Ad. 46. Purchased from Baarn labelled *P. ochraceum*. "Ad. 46, labelled *P. ochraceum*, appears to be a culture distributed from the BAINIER collection under this name. No description was published by BAINIER, but since the culture had reached various workers under the name, I have continued the name as attributed to BAINIER and added a description."
- (iii) Ad. 12. Isolated at Ardeer from the "sweet waters" of a glycerol still. "Ad. 12 had a series of characters which throw it so far out of harmony with

the scheme of classification in the lanose group that I described it as *P. lanoso-viride* THOM, n.sp., on p. 314 of my new book. It seemed necessary to make a place for this in the scheme of classification so that I have applied this name and designated the culture as 'Type.'"

- (iv) Ad. 33. Purchased from Baarn in 1925, labelled *P. glaucum*. "Ad. 33 is incorrectly named and should be *P. lanosum* WESTLING."
- (v) Ad. 60. Purchased from Baarn in 1925, labelled *P. virescens*. "Ad. 60, labelled *P. virescens*, presumably of BAINIER. BAINIER's report gives the conidia as 2.8 μ and from his various notes I could not justify identifying a deeply growing floccose or fasciculate form under this name. Ad. 60 is a floccose form, with conidia about 3 μ , in the section *Lanata* (of BIOURGE as amended by me) and with the morphology of *P. lanosum* of WESTLING and the colour of *P. lanosocæruleum* THOM. These species are close together in this group, anyway."

Subsection 2. *Lanata-zonata*.

- (i) Ad. 82. Isolated at Ardeer in 1926 from mouldy artificial leather cloth. "Ad. 82 is *P. commune* THOM or a nearly related strain."
- (ii) Ad. 19. Isolated at Ardeer from spoiled fuse. "Ad. 19 is apparently *P. fusco-glaucum* BIOURGE."

Section IV. *Lanata-divaricata*.

Ad. 32. Purchased from Baarn in 1925, labelled *P. lilacinum*. "Ad. 32 is correctly named as *P. lilacinum*." Ad. 37. Purchased from the British National Collection of Type Cultures in 1925, labelled *P. elongatum*, Catalogue Number 1719, and the BAINIER Collection, No. 444. "Ad. 37 is certainly not *P. elongatum* of the BAINIER Collection but is actually *P. lilacinum* or near it."

Section V. *Asymmetrica-funiculosa*.

Subsection 1. *Funiculosa-divaricata*.

- (i) Ad. 55. Purchased from Baarn in 1925, labelled *P. palitans* WESTLING. "Ad. 55 labelled *P. palitans*: my later notes identify this with ZALESKI's *P. Daleæ*." Ad. 116. Purchased from Baarn, labelled *P. lanosum* WESTLING. No carbon balance sheet was prepared for this species, but it is referred to on p. 130 of Part VII as producing kojic acid. "Ad 116 is *P. Daleæ* ZALESKI in quite a different section from *P. lanosum*. I believe that my identification here is more correct than Dr. WESTERDIJK's and that *P. lanosum* may justly be held in a separate group from *P. Daleæ*."
- (ii) Ad. 62. Isolated at Ardeer as a bench contamination. This species was kept because of the unusual salmon-pink reverse on CZAPEK-DOX medium. "Ad. 62 is one of the section including *P. intricatum* and is nearest to *P. Godlewskii* of ZALESKI."

Subsection 2. Funiculosa-typica.

- (i) Ad. 22 received from Mr. F. T. BROOKS of Cambridge, labelled *P. viridicatum*.
 "Ad. 22. Here my record is not very satisfactory. You are doubtless correct in turning down the first allocation. A later note of mine assigns it to a series with *P. psittacinum* THOM into which I finally put the organism I received from BIOURGE under the name *P. aureum* CORDA, which I could in no way accept as correctly named. In colour and initial habit these colonies do suggest *P. viridicatum* as indicated in my first letter."
- (ii) Ad. 8. Isolated at Ardeer in 1922 as a bench contamination. Ad. 4. Received via Cambridge, labelled *P. glaucum* (cake) and distributed in the first place by the British National Collection of Type Cultures, Catalogue No. 764. Ad. 5 received via Cambridge, labelled "*Penicillium* from tan liquor" and bearing the British National Collection Catalogue No. 1162. Ad. 54. Purchased from Baarn in 1925, labelled *P. patulum*, BAINIER. Ad. 18. Isolated at Ardeer from milk in which it produced a yellow colour with the formation of masses of crystals. Ad. 50. Purchased from Baarn, labelled *P. granulatum*. Ad. 59. Purchased from Baarn in 1925, labelled *P. baculatum*. Ad. 9. Isolated at Ardeer in 1922 from cheese. "I have placed Nos. Ad. 8, Ad. 4, Ad. 5, Ad. 54, Ad. 18, Ad. 50, Ad. 59 and Ad. 9, together with certain forms which I have encountered here, and covered them by the name *P. terrestre* JENSEN series, which amounts to digging up JENSEN's name for a soil series of *Penicillium* and applying it to this group of organisms, which have certain fundamental characteristics in harmony and minor differences in general appearance."

*Section VI. Fasciculata.**Subsection 1. Sclerotigena.*

- (i) Ad. 65. Received from Mr. F. T. BROOKS of Cambridge, labelled *Penicillium B.*, isolated from and pathogenic on gladioli culms. "Ad. 65 is *P. gladioli* MACHACEK. The same name was published independently by McCULLOCH and THOM but later in date."
- (ii) Ad. 84. Isolated at Ardeer in 1926 from a mouldy orange. Ad. 85. Isolated at Ardeer in 1926 from a mouldy orange. Ad. 86. Isolated at Ardeer in 1926 from a mouldy orange. No carbon balance sheet was prepared for Ad. 86. "Ad. 84, Ad. 85 and Ad. 86 appear to be *P. italicum*."

Subsection 2. Aeruginosa.

None.

Subsection 3. Viridicata.

- (i) Ad. 15. Isolated at Ardeer from a very acid cellulose fermentation, having a pH of 1-2. "Ad. 15 is a member of the *P. viridicatum* group, a composite of

many variants about another group of characteristics. It is perhaps more nearly placed with *P. verrucosum* DIERCKX (according to BOURGE) than with WESTLING's *P. viridicatum*."

- (ii) Ad. 76. Isolated at Ardeer from mouldy artificial leather cloth. Ad. 77. Isolated at Ardeer from the cooling water of a glycerol still. Ad. 83. Isolated at Ardeer from artificial leather cloth. Ad. 77 and 83 were not sent to Dr. THOM but we believe them to be very closely related to Ad. 76. "Ad. 76 is a member of the series *P. viridicatum*. Whether placing *P. viridicatum* and *P. verrucosum* (see Ad. 15) in a series has more than arbitrary value based upon superficial characters is left open in my own mind. They were put together as having fasciculation, zonation, and certain related shades of colour and habit. We have not made a quantitative study of them."

Subsection 4. Glauca.

Series Crustaceum. Ad. 16. Isolated at Ardeer from a lemon. "Ad. 16: by our records we had to purify the culture received but selected as the organism probably indicated, a gray-green, faintly zonate form, showing slight fasciculation at the growing margin and forming great *crusts* of conidia. For lack of a better name I have made this another *type* which I have called *P. crustosum* THOM (n. sp.). It is described on p. 399 of my book."

Series Restrictum.

None.

Series Expansum.

Ad. 1. Purchased from the British National Collection, labelled *P. expansum* LINK, Catalogue No. 593, THOM and CHURCH, Washington 4189. Ad. 58. Purchased from Baarn in 1925, labelled *P. biforme*. "Ad. 1 is probably correct to the series—*P. expansum*, but exactly which one of the series is less definite. Ad. 58, labelled *P. biforme*; our transfers show not a floccose form but a fasciculate species in the great series with *P. expansum*, perhaps as a strain near *P. elongatum* of DIERCKX."

Series Italicum. (See above.)

Series—p. 416 in THOM's '*Penicillia*.'

Ad. 51. Purchased from Baarn in 1925, labelled *P. Schneggii* BOAS. "Ad. 51, *P. Schneggii*, BOAS: I think this probably represents BOAS' material." This culture is described on p. 417 in THOM's book.

Series—Urticæ-patulum.

None.

Subsection 5. Coremiella.

Ad. 61. Purchased from Baarn in 1925, labelled *P. corymbiferum* WESTLING. Ad. 64. Received from Mr. F. T. BROOKS, labelled *Penicillium A.* Isolated from and pathogenic to narcissus bulbs. "Ad. 61: *P. corymbiferum*. Ad. 64, labelled *P. corymbiferum*; my transfer of this when first received was so complete a reproduction of the description, my notes, and my memory of the strain WESTLING sent me, that I marked it with the name and moved the discussion of it in the book to suit the new data obtained from a species not before seen for 15 years."

Subsection 6. Coremium.

None.

*III. Biverticillata-symmetrica.**Section I. Ascogena.*

- (i) Ad. 39. Purchased from the British National Collection, labelled *P. avellaneum*, Washington 4010.5, British Catalogue No. 984. "Ad. 39, *P. avellaneum* THOM and TURESSON: this culture is apparently correctly named."
- (ii) Ad. 20. Isolated at Ardeer from mouldy tobacco. "Ad. 20: so far I find the sulphur-yellow bodies (sclerotia) which you note in your record and have not been able to get ascospores out of them. This species falls nearer to *P. Kiliense* of WEIDEMANN than any other described form. Since WEIDEMANN's species were never available to anyone else, perhaps we may use the name legitimately since the rest of the data did not go far astray."
- (iii) Ad. 101. Purchased in 1927 from the American National Collection, labelled *P. spiculisporum* LEHMAN, Catalogue No. 1136. "Ad. 101 is probably correct as *P. spiculisporum*."

Section II. Coremigena.

Ad. 63. Purchased from the British National Collection, labelled *P. Duclauxi*, Washington 20, British Catalogue No. 587. Ad. 75. Isolated at Ardeer from sand. "Ad. 63 is correctly named as *P. Duclauxi* DELACROIX. This goes back to a culture received from DELACROIX himself just before his death. Ad. 75 is correctly named as *P. Duclauxi*."

*Section III. Luteo-virida.**Subsection IIIa. Funiculosa.*

- (i) *Luteo-viride-pinophilum* Series.—Ad. 41. Purchased in 1925 from the British Collection, labelled *P. pinophilum* HEDGCOCK. THOM and CHURCH, Washington, British Catalogue No. 1151. "Ad. 41 is correctly named as *P. pinophilum*."
- (ii) *P. funiculosum* Series.—None.
- (iii) *P. Herquei* Series.—Ad. 43. Purchased from Baarn in 1925, labelled *P. Herquei* BAINIER and SARTORY. "Ad. 43 is correctly named as *P. Herquei*."

Subsection IIIb. Luteo-purpurogena.

- (i) *P. rugulosum* Series.—Ad. 27. Purchased from the British National Collection in 1925, labelled *P. rugulosum* THOM, Catalogue No. 592, THOM and CHURCH, Washington 46. “Ad. 27, labelled *P. rugulosum* THOM: this is certainly a strain in the series; whether it is the original No. 46 or not looks doubtful, but let it stand.”
- (ii) *P. purpurogenum* Series.—Ad. 36. Purchased in 1925 from the British National Collection, labelled *P. purpurogenum* FLEROFF, Catalogue No. 586, Washington 17. “Ad. 36 is *P. purpurogenum* STOLL, or FLEROFF-STOLL if you prefer.”
- (iii) *P. luteum* Series (Non-ascosporic).—Ad. 30. Purchased from Baarn in 1925, labelled *P. luteum* ZUKAL. “Ad. 30 is apparently a member of the *P. luteum* group. In this case we also run up against a peculiar history, as you know. The original culture which I reported as *P. luteum*, No. 11, has been maintained for twenty years as an ascosporic form received from Prof. THAXTER, carried to Prof. WEHMER, labelled and agreed to by him as this species. The validity of this species is attacked by DERX and BOURGE. Several times in the period during which I have kept it in culture it has separated into two lines, one of which is pale in colour, very little yellow either above or below, and produces no ascospores. This form harmonizes with Ad. 30 as I have received it. This strain would, in a way, satisfy DERX’ contention that ascosporic forms in this series are produced by the conjugation of two strains which are separately non-ascosporic.”

Section IV. Biverticillata miscellanea.

- Ad. 45. Purchased from Baarn in 1925, labelled *P. vesiculosum* BAINIER. “Ad. 45, labelled *P. vesiculosum* BAINIER: though where it got the name I cannot say. I believe it to be identified correctly with *P. elongatum* BAINIER, but since that name was applied several years earlier by DIERCKX to a very different organism, some change had to be made. Hence, since it grows very slightly and scantily in certain media, I named it in my book as *P. tardum* THOM (see p. 486).”
- Ad. 47. Purchased from Baarn, labelled *P. claviforme* BAINIER. “Ad. 47 is actually another strain of *P. tardum* as in Ad. 45. There are several strains of this lot, some of which grow much more readily and profusely than the type.”

IV. Polyverticillata-symmetrica.

None.

Genus *Gliocladium* CORDA

- Ad. 38. Purchased in 1925 from the British National Collection, labelled *P. roseum* PAINE, Catalogue No. 1290. “Ad. 38: we could not get our transfers of this

mould to produce conidia, so that we do not know whether it has degenerated or what is the explanation. The general habit of the colony was about that of the *P. roseum* or *Gliocladium roseum* lot for forms."

Genus *Scopulariopsis* BAINIER.

- Ad. 40. Purchased from the British National Collection, labelled *P. brevicaule* SACCARDO, Catalogue No. 580, Washington 2. "Ad. 40, labelled *P. brevicaule*, is certainly one of the lot." Ad. 70. Purchased from PRIBRAM of Vienna in 1926, labelled *Citromyces B.* WEHMER. Ad. 72. Purchased in 1926 from PRIBRAM of Vienna, labelled *Citromyces citricus I* WEHMER. "Ad. 70 is some variety of *P. brevicaule*, or better, *Scopulariopsis*. Ad. 72 is another *Scopulariopsis* (*P. brevicaule*). My comment at this point is that your note indicates that you purchased them from PRIBRAM. I bought those same cultures and they were nearly all contaminated."

Genus *Pæcilomyces* BAINIER.

- Ad. 44. Purchased from Baarn in 1925, labelled *P. divaricatum*. "Ad. 44 is correctly named as *P. divaricatum* THOM = *Pæcilomyces varioti* BAINIER. or very nearly so."

Unnamed *Penicillium* Species.

- Ad. 3. Received from Cambridge and is supposed to be British National Collection Culture No. 589, labelled *P. chrysogenum* THOM, Washington 26. "Ad. 3, labelled *P. chrysogenum*, is either a degenerate or an entire or almost complete replacement of *P. chrysogenum* with a white sterile form." Ad. 57. Purchased from Baarn in 1925, labelled *P. variable* WEHMER. "Ad. 57, labelled *P. variable*, is the same kind of culture in our transfers as Ad. 3. It is a broadly spreading white organism with a few green fruits about the margin." THOM says on p. 411 of his book on the *Penicillia*: "Cultures received under the name (*P. variable*) from certain other German sources in 1924-1926 have been races of *P. chrysogenum*, hence the substitution of this organism for WEHMER's species has become complete in certain laboratories." The history of Ad. 3 and Ad. 57 is thus particularly interesting.
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Studies in the Biochemistry of Micro-organisms.

PART V.—*Quantitative examination by the carbon balance sheet method of the types of products formed from glucose by species of Fusarium.*

By JOHN HOWARD BIRKINSHAW, JOHN HENRY VICTOR CHARLES, HAROLD RAISTRICK,
and JOHN ALEXANDER ROBERTSON STOYLE.

In view of the encouraging results obtained from the preparation of carbon balance sheets for a number of species of *Aspergillus*, as reported in Part III, the work was extended to other groups and, by way of contrast, carbon balance sheets were prepared for a considerable number of species of *Fusarium*. It is evident from WOLLENWEBER'S "Monograph on the *Fusaria*" (see 'Phytopathology' (1913), Vol. 3, p. 24) that the classification of the *Fusaria* on morphological grounds is very difficult. It was hoped that, as a result of the preparation of carbon balance sheets, some biochemical classification on the lines of the *Aspergillus* group might be possible, and in order to ensure that the cultures used were authentic, almost the whole of those chosen for investigation were WOLLENWEBER'S own cultures purchased from the Centraalbureau voor Schimmelcultures at Baarn. The methods of investigation adopted were those described in Part II and applied to the *Aspergillus* group in Part III.

The following is the history of the species of *Fusarium* used in this work :—

- * (1) *F. viride* (LECHM.) WR., Catalogue No. Ag. 81. Purchased from Centraalbureau voor Schimmelcultures at Baarn.
- * (2) *F. solani* MART. var. *minus* WR., Catalogue No. Ag. 83. Purchased from Baarn.
- (3) *F. lini* BOLLEY, Catalogue No. Ag. 60. Purchased from British National Collection of Type Cultures, No. 1082.
- * (4) *F. Martii* APP. et WR., Catalogue No. Ag. 77. Purchased from Baarn.
- (5) *F. species*, Catalogue No. Ag. 80. Isolated at Ardeer from infected potato haulm.
- (6) *F. dianthi* PRILL. et DEL., Catalogue No. Ag. 59. Purchased from British National Collection of Type Cultures No. 1136.
- * (7) *F. uncinatum* WR., Catalogue No. Ag. 75. Purchased from Baarn.
- * (8) *F. trichothecioides* WR., Catalogue No. Ag. 73. Purchased from Baarn.
- * (9) *F. tubercularioides* (CORDA) SACC., Catalogue No. Ag. 72. Purchased from Baarn.
- * (10) *F. cæruleum* (LIB.) SACC., Catalogue No. Ag. 65. Purchased from Baarn.
- * (11) *F. salicis* FÜCK., Catalogue No. Ag. 69. Purchased from Baarn.
- * (12) *F. javanicum* KOORDERS, Catalogue No. Ag. 78. Purchased from Baarn.

- *(13) *F. falcatum* APP. et WR., Catalogue No. Ag. 82. Purchased from Baarn.
- *(14) *F. avenaceum* (FR.) SACC., Catalogue No. Ag. 76. Purchased from Baarn.
- (15) *F. sporotrichoides* SHERB., Catalogue No. Ag. 58. Purchased from British National Collection of Type Cultures, No. 1296.
- *(16) *F. sambucinum* FUCK., Catalogue No. Ag. 70. Purchased from Baarn.
- (17) *F. vasinfectum* ATK., Catalogue No. Ag. 71. Purchased from Baarn.
- *(18) *F. rhizophilum* CORDA, Catalogue No. Ag. 74. Purchased from Baarn.
- *(19) *F. metachroum* APP. et WR., Catalogue No. Ag. 68. Purchased from Baarn.
- (20) *F. species*, Catalogue No. Ag. 61. Isolated at Ardeer from a rotting potato.
- *(21) *F. orthoceras* APP. et WR., Catalogue No. Ag. 66. Purchased from Baarn.
- (22) *F. scirpi* LAMB et FAUTR., Catalogue No. Ag. 79. Purchased from Baarn.
- (23) *F. oxysporum* SCHLECHT, Catalogue No. Ag. 67. Purchased from Baarn.

Fifteen of the cultures in the above list which are marked with an asterisk were WOLLENWEBER's cultures purchased from Baarn.

The carbon balance sheets prepared for these species are given in Tables I and II.

Discussion of results obtained.

None of the species grew very well on the synthetic CZAPEK-DOX metabolism solution used, as is evidenced by the relatively small weights of mycelium obtained, compared with the corresponding figures for species of *Aspergillus*. It is significant, however, that in spite of the relatively small growth of mycelium, the amount of glucose metabolized was very considerable.

The only metabolic product produced in quantity by any of the *Fusaria* is in the group known as "volatile neutral compounds," and in one or two instances where investigation as to its nature took place, it was shown to be ethyl alcohol. The carbon balance sheets show, indeed, that, biochemically speaking, the *Fusaria* function in a very similar manner to the *Saccharomyces*, and in many cases the yields of alcohol formed from glucose by species of *Fusarium* compare favourably with the corresponding yields given by the best species of *Saccharomyces*. Thus, with *F. avenaceum* (Ag. 76), of 4.638 gm. of carbon as glucose metabolized, 2.453 gm. appear as "volatile neutral compounds" representing a yield of 52.9 per cent. This yield is even larger if, in calculating it, allowance is made for the carbon utilised by the fungus in building up its mycelium. Calculated on these lines, the yield is 55.1 per cent. Almost theoretical values could, no doubt, be obtained if ready-grown mycelia were used. The theoretical yield is 66.7 per cent., and the maximum yield obtained with species of *Saccharomyces* is about 60 per cent.

The property of the *Fusaria* of producing large quantities of alcohol from glucose might be turned to account technically in the production of alcohol from waste vegetable matter. Many species of *Fusarium* attack vegetable matter in the raw state without

| Species of <i>Fusarium</i> : | <i>F. viride</i> . | <i>F. solani</i> , var. <i>minus</i> . | <i>F. lini</i> . | <i>F. Martii</i> . | <i>F. species</i> (from potato). | <i>F. dianthi</i> . | <i>F. uncin-</i> <i>atum</i> . | <i>F. tricho-</i> <i>thecoides</i> . | <i>F. tuber-</i> <i>culari-</i> <i>oides</i> . | <i>F. cer-</i> <i>uleum</i> . | <i>F. salicis</i> . | <i>F. java-</i> <i>nicum</i> . |
|--|--------------------|--|------------------|--------------------|--|---------------------|-----------------------------------|---|--|----------------------------------|---------------------|-----------------------------------|
| Catalogue number: | Ag. 81 | Ag. 83 | Ag. 60 | Ag. 77 | Ag. 80 | Ag. 59 | Ag. 75 | Ag. 73 | Ag. 72 | Ag. 65 | Ag. 69 | Ag. 78 |
| Experiment number: | A 25 | A 27 | A 29 | A 21 | A 24 | A 3 | A 19 | A 18 | A 28 | A 9 | A 13 | A 22 |
| Incubation period in days: | 43 | 51 | 56 | 51 | 72 | 34 | 34 | 41 | 41 | 32 | 52 | 36 |
| <i>Carbon Balance Sheet.</i> | | | | | | | | | | | | |
| Carbon in solution (start) ... gm. | 4.901 | 4.901 | 4.901 | 4.901 | 4.901 | 5.018 | 5.018 | 5.018 | 4.901 | 5.018 | 5.018 | 4.901 |
| Carbon in H_2SO_4 ... | 0.003 | 0.005 | 0.009 | 0.010 | 0.002 | 0.020 | 0.019 | 0.011 | 0.017 | 0.016 | 0.015 | 0.019 |
| " in CO_2 ... | 1.053 | 1.204 | 1.652 | 1.361 | 1.162 | 1.654 | 1.487 | 1.279 | 1.532 | 1.380 | 1.178 | 1.613 |
| " in mycelium ... | 0.382 | 0.430 | 0.450 | 0.267 | 0.385 | 0.373 | 0.216 | 0.137 | 0.195 | 0.392 | 0.107 | 0.285 |
| " in solution (end) ... | 3.439 | 3.175 | 2.642 | 3.161 | 3.317 | 2.794 | 3.143 | 3.461 | 3.077 | 3.093 | 3.577 | 2.802 |
| " accounted for ... | 4.877 | 4.814 | 4.753 | 4.799 | 4.866 | 4.841 | 4.865 | 4.888 | 4.821 | 4.881 | 4.877 | 4.719 |
| " accounted for ... per cent. | 99.5 | 98.2 | 97.0 | 97.9 | 99.3 | 96.5 | 97.0 | 97.4 | 98.4 | 97.3 | 97.2 | 96.3 |
| <i>Analysis of Solution.</i> | | | | | | | | | | | | |
| Carbon in residual glucose | 2.410 | 1.948 | 0.945 | 1.428 | 2.564 | 0.712 | 0.380 | 0.744 | 0.086 | 0.641 | 1.035 | 0.143 |
| " in CO_2 in solution ... gm. | 0.005 | 0.005 | 0.000 | 0.004 | 0.008 | 0.011 | 0.017 | 0.021 | 0.006 | 0.012 | 0.018 | 0.017 |
| " in volatile acids ... | 0.198 | 0.212 | 0.175 | 0.130 | 0.109 | 0.088 | 0.010 | 0.053 | 0.070 | 0.018 | 0.097 | 0.034 |
| " in non-volatile acids ... | 0.054 | 0.067 | 0.078 | 0.076 | 0.057 | 0.084 | 0.251 | 0.247 | 0.241 | 0.237 | 0.187 | 0.169 |
| " in volatile neutral compounds ... | 0.368 | 0.488 | 1.006 | 1.183 | 0.109 | 1.580 | 2.228 | 2.120 | 2.362 | 1.870 | 2.126 | 2.246 |
| " in synthetic compounds ... | 0.074 | 0.144 | 0.124 | 0.132 | 0.059 | 0.151 | 0.123 | 0.082 | 0.183 | 0.132 | 0.043 | 0.202 |
| Total carbon accounted for ... | 3.109 | 2.864 | 2.328 | 2.953 | 2.906 | 2.626 | 3.009 | 3.267 | 2.948 | 2.910 | 3.506 | 2.811 |
| " " in solution ... | 3.439 | 3.175 | 2.642 | 3.161 | 3.317 | 2.794 | 3.143 | 3.461 | 3.077 | 3.093 | 3.577 | 2.802 |
| Carbon unaccounted for (by difference),, | 0.330 | 0.311 | 0.314 | 0.208 | 0.411 | 0.168 | 0.134 | 0.194 | 0.129 | 0.183 | 0.071 | Surplus of 0.009 |
| <i>Residual Glucose.</i> | | | | | | | | | | | | |
| Glucose (by polarimeter) ... per cent. | 1.308 | 1.040 | 0.504 | 0.726 | 1.383 | 0.428 | 0.199 | 0.320 | 0.018 | 0.378 | 0.493 | 0.139 |
| " (SHAFFER-HARTMANN) ... | 1.205 | 0.974 | 0.473 | 0.714 | 1.282 | 0.356 | 0.190 | 0.372 | 0.043 | 0.320 | 0.517 | 0.072 |
| " (WOOD-OST) ... | 1.260 | 1.052 | 0.496 | 0.744 | 1.286 | 0.369 | — | 0.373 | — | 0.311 | 0.508 | — |
| " (by alkaline iodine) ... | 1.257 | 1.035 | 0.522 | 0.769 | 1.361 | 0.384 | 0.315 | 0.539 | 0.266 | 0.454 | 0.717 | 0.261 |
| <i>Acids.</i> | | | | | | | | | | | | |
| Titration (N/1 acid) ... c.c. | 4.9 | 4.8 | 4.1 | 4.7 | 1.6 | 1.0 | 7.1 | 6.5 | 5.4 | 6.1 | 5.2 | 0.9 |
| Volatile acids (N/1 acid) ... | 9.54 | 9.07 | 8.20 | 7.00 | 5.69 | 0.33 | 1.03 | 1.86 | 2.92 | 0.26 | 3.30 | 1.32 |
| Barium salts (weight) ... gm. | 1.045 | 1.089 | 0.925 | 0.698 | 0.562 | 0.027 | 0.027 | 0.204 | 0.307 | 0.053 | 0.409 | 0.121 |
| Calcium salts (weight) ... | — | 0.329 | 0.289 | 0.537 | 0.193 | 0.353 | 1.162 | 1.008 | 0.961 | 1.131 | 0.783 | 0.831 |
| Volume of oxygen absorbed ... c.c. | 1362 | 1557 | 1814 | 1274 | 1838 | 1678 | 630 | 463 | 564 | 705 | 345 | 784 |
| Respiration coefficient ... | 1.45 | 1.45 | 1.70 | 2.00 | 1.19 | 1.85 | 4.46 | 5.24 | 5.10 | 3.69 | 6.45 | 3.88 |
| Mycelium (weight) ... gm. | 0.732 | 0.849 | 0.918 | 0.505 | 0.709 | 0.718 | 0.407 | 0.258 | 0.366 | 0.762 | 0.199 | 0.514 |
| " (carbon) ... per cent. | 52.2 | 50.6 | 49.0 | 53.0 | 54.3 | 51.9 | 53.0 | 53.1 | 53.2 | 51.4 | 53.9 | 55.5 |

TABLE II.—Carbon balance sheets for various species of *Fusarium*.

| Species of <i>Fusarium</i> : | <i>F. falcatum</i> , | <i>F. avenaceum</i> , | <i>F. sporotrichoides</i> , | <i>F. sambucinum</i> , | <i>F. vesinifectum</i> , | <i>F. rhizophitum</i> , | <i>F. metachromum</i> , | <i>F. species</i> , | <i>F. orthoceras</i> , | <i>F. scirpi</i> , | <i>F.oryz-sporum</i> , |
|--|----------------------|-----------------------|-----------------------------|------------------------|--------------------------|-------------------------|-------------------------|---------------------|------------------------|--------------------|------------------------|
| Catalogue number : | Ag. 82 | Ag. 76 | Ag. 58 | Ag. 70 | Ag. 71 | Ag. 74 | Ag. 68 | Ag. 61 | Ag. 66 | Ag. 79 | Ag. 67 |
| Experiment number : | A 26 | A 20 | A 4 | A 14 | A 15 | A 17 | A 12 | A 6 | A 10 | A 23 | A 11 |
| Incubation period in days : | 56 | 39 | 67 | 45 | 58 | 70 | 66 | 71 | 40 | 33 | 50 |
| <i>Carbon Balance Sheet.</i> | | | | | | | | | | | |
| Carbon in solution (start) ... gm. | 4.901 | 4.901 | 5.018 | 5.018 | 5.018 | 5.018 | 5.018 | 5.018 | 5.018 | 4.901 | 5.018 |
| Carbon in H ₂ SO ₄ ... | 0.021 | 0.018 | 0.028 | 0.034 | 0.015 | 0.015 | 0.026 | 0.033 | 0.024 | 0.023 | 0.031 |
| " in CO ₂ ... | 1.550 | 1.536 | 1.485 | 1.617 | 1.486 | 1.104 | 1.240 | 1.273 | 1.831 | 1.826 | 1.965 |
| " in mycelium... .. | 0.281 | 0.187 | 0.386 | 0.208 | 0.276 | 0.211 | 0.199 | 0.145 | 0.207 | 0.453 | 0.269 |
| " in solution (end) ... | 2.989 | 3.085 | 2.980 | 2.974 | 3.118 | 3.634 | 3.463 | 3.490 | 2.851 | 2.452 | 2.589 |
| " accounted for ... | 4.841 | 4.826 | 4.879 | 4.833 | 4.895 | 4.964 | 4.928 | 4.941 | 4.913 | 4.754 | 4.854 |
| " accounted for ... per cent. | 98.8 | 98.5 | 97.2 | 96.3 | 97.6 | 99.0 | 98.2 | 98.5 | 97.9 | 97.0 | 96.7 |
| <i>Analysis of Solution.</i> | | | | | | | | | | | |
| Carbon in residual glucose ... gm. | 0.056 | 0.263 | 1.158 | 0.129 | 0.892 | 1.838 | 1.530 | 1.575 | 0.077 | 0.025 | 0.004 |
| " in CO ₂ in solution ... | 0.010 | 0.019 | 0.003 | 0.013 | 0.015 | 0.008 | 0.005 | 0.009 | 0.018 | 0.021 | 0.031 |
| " in volatile acids ... | 0.035 | 0.027 | 0.033 | 0.032 | 0.029 | 0.018 | 0.009 | 0.018 | 0.025 | 0.033 | 0.021 |
| " in non-volatile acids ... | 0.065 | 0.127 | 0.140 | 0.125 | 0.112 | 0.070 | 0.078 | 0.084 | 0.107 | 0.102 | 0.117 |
| " in volatile neutral compounds ... | 2.362 | 2.453 | 1.389 | 2.464 | 1.846 | 1.528 | 1.684 | 1.612 | 2.332 | 2.078 | 2.165 |
| " in synthetic compounds ... | 0.191 | 0.098 | 0.092 | 0.104 | 0.079 | 0.129 | 0.063 | 0.080 | 0.095 | 0.130 | 0.058 |
| Total carbon accounted for ... | 2.719 | 2.987 | 2.815 | 2.867 | 2.973 | 3.591 | 3.369 | 3.378 | 2.654 | 2.389 | 2.396 |
| " in solution ... | 2.989 | 3.085 | 2.980 | 2.974 | 3.118 | 3.634 | 3.463 | 3.490 | 2.851 | 2.452 | 2.589 |
| Carbon unaccounted for (by difference), | 0.270 | 0.098 | 0.165 | 0.107 | 0.145 | 0.043 | 0.094 | 0.112 | 0.197 | 0.063 | 0.193 |
| <i>Residual Glucose.</i> | | | | | | | | | | | |
| Glucose (by polarimeter) ... per cent. | 0.037 | 0.219 | 0.748 | 0.029 | 0.460 | 0.973 | 0.767 | 0.767 | 0.099 | 0.106 | 0.018 |
| " (SHAFFER-HARTMAN) ... | 0.028 | 0.131 | 0.579 | 0.064 | 0.446 | 0.919 | 0.765 | 0.787 | 0.039 | 0.013 | 0.002 |
| " (WOOD-OET) ... | — | — | 0.598 | — | 0.444 | 0.950 | 0.778 | 0.803 | — | — | — |
| " (by alkaline iodine) ... | 0.112 | 0.215 | 0.740 | — | 0.550 | 0.966 | 0.829 | 0.836 | — | 0.093 | — |
| <i>Acids.</i> | | | | | | | | | | | |
| Titration (N/1 acid) ... c.c. | 4.1 | 3.2 | 2.3 | 1.9 | 1.5 | 1.0 | 0.9 | 0.8 | 0.3 | 0.7 | Decrease of 1.3 |
| Volatile acids (N/1 acid) ... | 2.15 | 1.47 | 0.95 | 1.52 | 1.58 | 1.33 | 0.73 | 0.66 | 0.87 | 2.04 | 1.96 |
| Barium salts (weight) ... gm. | 0.186 | 0.135 | 0.093 | 0.133 | 0.125 | 0.074 | 0.059 | 0.053 | 0.051 | 0.177 | 0.180 |
| Calcium salts (weight) ... | 0.675 | 0.751 | 0.655 | 0.689 | 0.656 | 0.486 | 0.453 | 0.361 | 0.526 | 0.515 | 0.684 |
| Volume of oxygen absorbed ... c.c. | 623 | 532 | 1465 | 641 | 1018 | 574 | 699 | 923 | 1047 | 1090 | 1339 |
| Respiration coefficient ... | 4.67 | 5.46 | 1.90 | 4.74 | 2.76 | 3.61 | 3.32 | 2.59 | 3.30 | 3.17 | 2.78 |
| Mycelium (weight) ... gm. | 0.530 | 0.361 | 0.677 | 0.375 | 0.456 | 0.346 | 0.358 | 0.278 | 0.384 | 0.877 | 0.518 |
| " (carbon) ... per cent. | 53.0 | 51.9 | 57.0 | 55.3 | 60.5 | 61.2 | 55.7 | 52.1 | 53.8 | 51.7 | 51.9 |

preliminary hydrolysis by some other agent, and it is conceivable that, since they also ferment pentoses with the production of alcohol, some species of *Fusarium* might prove to be a suitable organism for solving the problem of obtaining alcohol from waste vegetable material.

Perusal of the balance sheets shows that, unlike the *Aspergilli*, no classification of the *Fusaria* is possible on the lines of production or non-production of alcohol ("volatile neutral compounds") since of the twenty-three species examined all give appreciable quantities of alcohol, and almost all give very good yields indeed. However, though no satisfactory classification of the *Fusaria* appears to be possible from the carbon balance sheets presented, it is possible to separate a few species which have rather unusual characteristics. For this reason the different species examined are arranged in the two tables in the following manner:—The species are arranged in order of decreasing acid production, the first members of Table I being those which give rise to the largest amounts of "volatile acids," and immediately succeeding them come the species which give rise to "non-volatile acids," so that at the one end of the table are placed those species giving the largest amount of volatile acids, while at the other end are those species which give very little acidity either of a volatile or a non-volatile nature. This arrangement enables one to effect a rough though not very satisfactory separation of the species.

Thus the following species *F. viride*, Ag. 81; *F. solani* var. *minus*, Ag. 83; *F. lini*, Ag. 60; *F. Martii*, Ag. 77; *F. species* (from potato), Ag. 80, and *F. dianthi*, Ag. 59, seem to form a group having the following characteristics:—None of them produces any appreciable amount of "non-volatile acids," while all produce relatively large amounts of "volatile acids." This is, of course, in direct contrast to the majority of species of *Aspergillus*, in which the acidity produced is almost entirely of the "non-volatile acid" type. The nature of this volatile acid has not been investigated, but if it were desired to do so it is obvious that the two species, Ag. 81 and Ag. 83, would be the most suitable for the purpose, since the "carbon in volatile acids" is 0.198 and 0.212 gm. respectively, corresponding to yields of 8.0 and 7.2 per cent. of the sugar fermented. Each of the five species in this sub-group also gives rise to moderate amounts of "carbon unaccounted for," though in no case is this very large, since the maximum is 0.411 gm. with *F. species*, Ag. 80, corresponding to 18 per cent. of the sugar fermented.

Further indication that these species may be regarded as a separate sub-group is the fact that while all of them give rise to larger weights of mycelium than the majority of the other species of *Fusarium* investigated, the amount of glucose metabolized by them is small. The amount of "volatile neutral compounds" formed by these five species is also very much less than the average for the remainder, and their respiration coefficients are very much lower than those of the other species.

Another sub-group might be formed of the following species:—*F. uncinatum*, Ag. 75; *F. trichothecioides*, Ag. 73; *F. tubercularioides*, Ag. 72; *F. caeruleum*, Ag. 65;

F. salicis, Ag. 69; and *F. javanicum*, Ag. 78, having the following characteristics:—The acidity produced by these species is, in contrast to the first sub-group, largely of the non-volatile acid type, and in this respect they resemble the *Aspergilli* more than any other species of *Fusarium*. The amount of titratable acidity or of non-volatile acids is, however, not very large, although it is sufficiently so to mark these species as being somewhat different from the remainder of the species of *Fusarium* investigated. All species in this group produce large amounts of volatile neutral compounds, and all of them have very large respiration coefficients. It will be noted that with almost all the species in this group, and also with *F. falcatum*, Ag. 82; *F. avenaceum*, Ag. 76; and *F. sporotrichoides*, Ag. 58, which may be regarded as the connecting link between this group and the remainder of the species of *Fusarium*, there is in every case a very marked difference between the percentage of residual glucose as estimated by alkaline iodine and by the SHAFFER-HARTMANN method. It is very probable that this is due to the characteristic which we have shown these species of *Fusarium* to possess, of reducing the sodium nitrate present in the CZAPEK-DOX medium to ammonia, which, in presence of alkaline iodine, forms iodine-substituted nitrogen compounds (probably nitrogen tri-iodide), hence giving results for glucose present which are far too high.

The remainder of the species of *Fusarium* which are given in Table II have no very special characteristics, except that they all give rise to very large amounts of volatile neutral compounds, and all have very high respiration coefficients.

Summary.

A quantitative examination has been made by the carbon balance sheet method, described in Part II, of the types of products formed from glucose by 23 species of *Fusarium*. These carbon balance sheets are presented in two tables from which a rough biochemical classification of the *Fusaria* can be made, but this is not nearly so satisfactory as that evolved for the *Aspergillus* group.

The main biochemical characteristic of species of *Fusarium* is that, like the *Saccharomyces*, they give rise to large amounts of alcohol from glucose. It is suggested that, since many species of *Fusarium* grow readily on waste vegetable materials, it may be possible to solve the problem of producing alcohol from waste vegetable matter by the use of a selected species of *Fusarium*.

Studies in the Biochemistry of Micro-organisms.

PART VI.—*Quantitative examination by the carbon balance sheet method of the types of products formed from glucose by miscellaneous species of fungi.*

By JOHN HOWARD BIRKINSHAW, JOHN HENRY VICTOR CHARLES, ARTHUR CLEMENT HETHERINGTON and HAROLD RAISTRICK.

In view of the encouraging results obtained from the preparation of carbon balance sheets, particularly for species of *Aspergillus* and *Penicillium*, the work was now extended to include a number of varied species of fungi belonging to many other genera. The carbon balance sheets for these miscellaneous species are given in this paper. The conditions of working adopted were exactly the same as those previously used and described in detail in Parts II and III. The results are arranged under the different genera according to the species of fungi investigated, and include the following :—

Class : ASCOMYCETES.

Genus 1. *Sordaria*, 1 species.

Genus 2. *Chaetomium*, 1 species.

Genus 3. *Sclerotinia* (conidial form = *Botrytis*), 1 species.

Class : BASIDIOMYCETES.

Genus 1. *Ustilago*, 2 species.

FUNGI IMPERFECTI.

Order : HYPHOMYCETALES.

Family : MONILIACEÆ.

Genus 1. *Eidamia*, 2 species.

Genus 2. *Sporotrichum*, 2 species.

Genus 3. *Trichoderma*, 2 species.

Genus 4. *Cephalothecium*, 1 species.

Family : DEMATIACEÆ.

Genus 1. *Cladosporium*, 5 species.

Genus 2. *Helminthosporium*, 6 species.

Genus 3. *Heterosporium*, 2 species.

Genus 4. *Alternaria*, 3 species.

Genus 5. *Fumago*, 2 species.

Genus 6. *Clasterosporium*, 2 species.

Genus 7. *Rhacodium*, 1 species.

Family: STILBACEÆ.

Genus 1. *Stysanus*, 1 species.

Family: TUBERCULARIACEÆ.

Genus 1. *Fusarium* (see Part V).

Genus 2. *Epicoccum*, 2 species.

Class: ASCOMYCETES.

The following is the history of the species included in this class:—

Genus 1. *Sordaria*, 1 species.

Sordaria species, Catalogue No. Ae. 11. Isolated at Ardeer from decaying cotton sludge from methane plant.

Genus 2. *Chaetomium*, 1 species.

Chaetomium species, Catalogue No. Ae. 2. Isolated by Mr. F. T. BROOKS, of Cambridge, from waste paper.

Genus 3. *Sclerotinia* (conidial form).

Botrytis cinerea PERSOON, Catalogue No. Ae. 4. Isolated at Ardeer from decaying rosebud.

The carbon balance sheets of these three species are given in Table I.

None of these species grows particularly well on the CZAPEK-DOX solution used. The three carbon balance sheets show nothing of any particular biochemical interest except in the case of *Sordaria* species. This fungus gives rise to comparatively large amounts of volatile neutral compounds (alcohol) from glucose and has a fairly high respiration coefficient (1.56). In all other respects the main function of these three species seems to be to convert glucose into carbon dioxide.

Class: BASIDIOMYCETES.

Genus. *Ustilago*, 2 species.

(1) *Ustilago Mayidis* (D.C.) CORDA, Catalogue No. Af. 8. Purchased from Centraalbureau voor Schimmelcultures at Baarn.

(2) *Ustilago avenæ* (PERS.) JENSEN, Catalogue No. Af. 7. Purchased from Baarn.

The carbon balance sheets for these two species are given in Table II.

These two species, and in particular *Ustilago avenæ*, were exceptionally difficult to cultivate on CZAPEK-DOX solution. Their carbon balance sheets are uninteresting, as they do not give rise to appreciable amounts of any metabolic products except carbon dioxide.

TABLE I.—Carbon balance sheets for three species of *Ascomycetes*.

| Species of <i>Ascomycetes</i> : | <i>Sordaria</i> species. | <i>Chaetomium</i> species. | <i>Sclerotinia</i> . |
|---|-----------------------------|-------------------------------|----------------------|
| Catalogue number : | Ae. 11 | Ae. 2 | Ae. 4 |
| Experiment number : | E 1 | E 2 | E 3 |
| Incubation period in days : | 90 | 70 | 86 |
| <i>Carbon Balance Sheet.</i> | | | |
| Carbon in solution (start) gm. | 4.901 | 5.043 | 5.043 |
| Carbon in H ₂ SO ₄ " | 0.016 | nil | 0.002 |
| " in CO ₂ " | 1.641 | 1.294 | 1.680 |
| " in mycelium " | 0.364 | 0.671 | 1.099 |
| " in solution (end) " | 2.747 | 2.940 | 2.107 |
| " accounted for " | 4.768 | 4.905 | 4.888 |
| " accounted for per cent. | 97.3 | 97.3 | 96.9 |
| <i>Analysis of Solution.</i> | | | |
| Carbon in residual glucose gm. | 1.251 | 2.624 | 1.574 |
| " in CO ₂ in solution " | 0.009 | 0.039 | 0.001 |
| " in volatile acids " | 0.005 | — | 0.009 |
| " in non-volatile acids " | 0.039 | 0.130 | 0.102 |
| " in volatile neutral compounds " | 1.156 | 0.001 | 0.015 |
| " in synthetic compounds " | 0.050 | 0.077 | 0.064 |
| Total carbon accounted for " | 2.510 | 2.871 | 1.765 |
| " " in solution " | 2.747 | 2.940 | 2.107 |
| Carbon unaccounted for (by difference) " | 0.237 | 0.069 | 0.342 |
| <i>Residual Glucose.</i> | | | |
| Glucose (by polarimeter) per cent. | 0.734 | 1.430 | 0.826 |
| " (SHAFFER-HARTMANN) " | 0.625 | 1.312 | 0.787 |
| " (WOOD-OST)... .. " | 0.603 | 1.328 | 0.744 |
| " (by alkaline iodine) " | 0.653 | 1.380 | 0.813 |
| <i>Acids.</i> | | | |
| Titration (N/1 acid) c.c. | 0.2 | Decrease of 0.8 | 0.6 |
| Volatile acids (N/1 acid) " | — | — | 0.32 |
| Barium salts (weight) gm. | 0.038 | — | 0.021 |
| Calcium salts (weight) " | 0.295 | 0.782 | 0.457 |
| Volume of oxygen absorbed c.c. | 1974 | 2049 | 2739 |
| Respiration coefficient " | 1.56 | 1.22 | 1.15 |
| Mycelium (weight) gm. | 0.730 | 1.264 | 1.903 |
| " (carbon) per cent. | 49.8 | 53.1 | 57.8 |

TABLE II.—Carbon balance sheets for species of *Ustilago*.

| Species of <i>Ustilago</i> : | | | | | | <i>U. Mayidis.</i> | <i>U. avenæ.</i> |
|--|-----|-----|-----|-----|-----------|--------------------|--------------------|
| Catalogue number : | | | | | | Af. 8 | Af. 7 |
| Experiment number : | | | | | | D 1 | D 2 |
| Incubation period in days : | | | | | | 80 | 124 |
| <i>Carbon Balance Sheet.</i> | | | | | | | |
| Carbon in solution (start) | ... | ... | ... | ... | gm. | 4.901 | 5.043 |
| Carbon in H ₂ SO ₄ | ... | ... | ... | ... | " | Nil | 0.001 |
| " in CO ₂ ... | ... | ... | ... | ... | " | 1.440 | 0.933 |
| " in mycelium | ... | ... | ... | ... | " | 0.878 | 0.526 |
| " in solution (end) | ... | ... | ... | ... | " | 2.485 | 3.416 |
| " accounted for | ... | ... | ... | ... | " | 4.803 | 4.876 |
| " accounted for | ... | ... | ... | ... | per cent. | 98.0 | 96.7 |
| <i>Analysis of Solution.</i> | | | | | | | |
| Carbon in residual glucose | ... | ... | ... | ... | gm. | 1.994 | 3.002 |
| " in CO ₂ in solution | ... | ... | ... | ... | " | 0.005 | 0.002 |
| " in volatile acids | ... | ... | ... | ... | " | 0.022 | Nil |
| " in non-volatile acids | ... | ... | ... | ... | " | 0.119 | 0.170 |
| " in volatile neutral compounds | ... | ... | ... | ... | " | 0.006 | Nil |
| " in synthetic compounds | ... | ... | ... | ... | " | 0.106 | 0.081 |
| Total carbon accounted for | ... | ... | ... | ... | " | 2.252 | 3.255 |
| " " in solution | ... | ... | ... | ... | " | 2.485 | 3.416 |
| Carbon unaccounted for (by difference) | ... | ... | ... | ... | " | 0.233 | 0.161 |
| <i>Residual Glucose.</i> | | | | | | | |
| Glucose (by polarimeter) | ... | ... | ... | ... | per cent. | 1.046 | 1.602 |
| " (SHAFFER-HARTMANN) | ... | ... | ... | ... | " | 0.997 | 1.501 |
| " (WOOD-OST) | ... | ... | ... | ... | " | 1.016 | 1.523 |
| " (by alkaline iodine) | ... | ... | ... | ... | " | 1.085 | 1.558 |
| <i>Acids.</i> | | | | | | | |
| Titration (N/1 acid) | ... | ... | ... | ... | c.c. | Decrease of 0.2 | Decrease of 0.9 |
| Volatile acids (N/1 acid) | ... | ... | ... | ... | " | 1.26 | 0.98 |
| Barium salts (weight) | ... | ... | ... | ... | gm. | 0.088 | 0.007 |
| Calcium salts (weight) | ... | ... | ... | ... | " | 0.580 | 0.785 |
| Volume of oxygen absorbed | ... | ... | ... | ... | c.c. | 2309 | 1734 |
| Respiration coefficient | ... | ... | ... | ... | ... | 1.17 | 1.01 |
| Mycelium (weight) | ... | ... | ... | ... | gm. | 1.561 | 1.012 |
| " (carbon) | ... | ... | ... | ... | per cent. | 56.2 | 52.0 |

FUNGI IMPERFECTI.

Order : HYPHOMYCETALES.

Family : MONILIACEÆ.

Genus 1. *Eidamia*, 2 species.

- (1) *Eidamia viridescens* HORNE et WILLIAMSON, Catalogue No. Ac. 75. Received from Dr. A. S. HORNE, Imperial College of Science and Technology.
- (2) *Eidamia catenulata* HORNE et WILLIAMSON, Catalogue No. Ac. 76. Received from Dr. HORNE.

The carbon balance sheets for these two species are given in Table III.

The general characteristics of the above species of *Eidamia* are given by HORNE (A. S.), and WILLIAMSON (H. S.) in a paper on "The Morphology and Physiology of the Genus *Eidamia*," 'Annals of Botany,' Vol. 37 (1923), p. 393. To quote from their paper, p. 393, "The genus *Eidamia* was founded by LINDAU to include fungi which bear a general resemblance to *Aspergillus*, but differ from it in possessing not only conidia, but also spores of a second type." This is particularly interesting since the carbon balance sheets for both species are of the type associated with certain species of *Aspergillus*. The production of large amounts of volatile neutral compounds, with correspondingly high respiration coefficients, absence of volatile acids, production of moderate amounts of titratable acidity, together with moderate amounts of carbon in the form of non-volatile acids, and a moderate value for "carbon unaccounted for," all suggest the type of carbon balance sheet associated with *A. clavatus*, or some strains of *A. niger*. It is also of interest to note that these two different species of *Eidamia* have similar types of carbon balance sheets.

Genus 2. *Sporotrichum*, 2 species.

- (1) *Sporotrichum carneolum*, Catalogue No. Ag. 23. Isolated from meat by Mr. F. T. BROOKS, of Cambridge.
- (2) *Sporotrichum bombycinum* (CORDA) RAB., Catalogue No. Ag. 25. Received from Baarn via Mr. F. T. BROOKS, of Cambridge.

The carbon balance sheets for these two species, which are given in Table IV, are both of the same type. Neither species gives rise to an appreciable amount of any metabolic product other than CO₂, and both of them produce an actual decrease in the original acidity of the medium. They both grow quite well on the CZAPEK-DOX medium used, but require a considerable time to metabolize even a moderate amount of glucose.

TABLE III.—Carbon balance sheets for species of *Eidamia*.

| Species of <i>Eidamia</i> : | | | | | | <i>E. viridescens.</i> | <i>E. catenulata.</i> |
|--|-----|-----|-----|-----|-----------|------------------------|-----------------------|
| Catalogue number : | | | | | | Ac. 75 | Ac. 76 |
| Experiment number : | | | | | | 102 | 103 |
| Incubation period in days : | | | | | | 45 | 35 |
| <i>Carbon Balance Sheet.</i> | | | | | | | |
| Carbon in solution (start) | ... | ... | ... | ... | gm. | 4.834 | 4.834 |
| Carbon in H ₂ SO ₄ | ... | ... | ... | ... | ... | 0.013 | 0.006 |
| " in CO ₂ ... | ... | ... | ... | ... | ... | 1.802 | 1.063 |
| " in mycelium | ... | ... | ... | ... | ... | 0.583 | 0.443 |
| " in solution (end) | ... | ... | ... | ... | ... | 2.376 | 3.168 |
| " accounted for | ... | ... | ... | ... | ... | 4.774 | 4.682 |
| " accounted for | ... | ... | ... | ... | per cent. | 98.8 | 96.9 |
| <i>Analysis of Solution.</i> | | | | | | | |
| Carbon in residual glucose | ... | ... | ... | ... | gm. | 0.777 | 1.812 |
| " in CO ₂ in solution | ... | ... | ... | ... | ... | 0.009 | 0.011 |
| " in volatile acids | ... | ... | ... | ... | ... | Nil | Nil |
| " in non-volatile acids | ... | ... | ... | ... | ... | 0.254 | 0.141 |
| " in volatile neutral compounds | ... | ... | ... | ... | ... | 0.952 | 0.839 |
| " in synthetic compounds | ... | ... | ... | ... | ... | 0.125 | 0.061 |
| Total carbon accounted for | ... | ... | ... | ... | ... | 2.117 | 2.864 |
| " " in solution | ... | ... | ... | ... | ... | 2.376 | 3.168 |
| Carbon unaccounted for (by difference) | ... | ... | ... | ... | ... | 0.259 | 0.304 |
| <i>Residual Glucose.</i> | | | | | | | |
| Glucose (by polarimeter) | ... | ... | ... | ... | per cent. | 0.367 | 0.911 |
| " (SHAFFER-HARTMANN) | ... | ... | ... | ... | ... | 0.388 | 0.906 |
| " (WOOD-OST) | ... | ... | ... | ... | ... | 0.388 | 0.978 |
| " (by alkaline iodine) | ... | ... | ... | ... | ... | 0.425 | 0.963 |
| <i>Acids.</i> | | | | | | | |
| Titration (N/1 acid) | ... | ... | ... | ... | c.c. | 4.3 | 2.7 |
| Volatile acids (N/1 acid) | ... | ... | ... | ... | ... | Nil | 0.44 |
| Barium salts (weight) | ... | ... | ... | ... | gm. | Nil | 0.008 |
| Calcium salts (weight) | ... | ... | ... | ... | ... | 1.027 | 0.621 |
| Volume of oxygen absorbed | ... | ... | ... | ... | c.c. | 2129 | 1190 |
| Respiration coefficient | ... | ... | ... | ... | ... | 1.59 | 1.69 |
| Mycelium (weight) | ... | ... | ... | ... | gm. | 1.146 | 0.901 |
| " (carbon) | ... | ... | ... | ... | per cent. | 50.9 | 49.4 |

TABLE IV.—Carbon balance sheets for species of *Sporotrichum*.

| Species of <i>Sporotrichum</i> : | | | | | | <i>S. carneolum.</i> | <i>S. bombycinum.</i> |
|--|-----|-----|-----|-----|-----------|----------------------|-----------------------|
| Catalogue number : | | | | | | Ag. 23 | Ag. 25 |
| Experiment number : | | | | | | C 2 | C 3 |
| Incubation period in days : | | | | | | 83 | 82 |
| <i>Carbon Balance Sheet.</i> | | | | | | | |
| Carbon in solution (start) | ... | ... | ... | ... | gm. | 4.901 | 4.901 |
| Carbon in H ₂ SO ₄ | ... | ... | ... | ... | „ | 0.001 | 0.001 |
| „ in CO ₂ ... | ... | ... | ... | ... | „ | 1.715 | 1.192 |
| „ in mycelium | ... | ... | ... | ... | „ | 0.780 | 1.041 |
| „ in solution (end) | ... | ... | ... | ... | „ | 2.303 | 2.439 |
| „ accounted for | ... | ... | ... | ... | „ | 4.799 | 4.673 |
| „ accounted for | ... | ... | ... | ... | per cent. | 97.9 | 95.4 |
| <i>Analysis of Solution.</i> | | | | | | | |
| Carbon in residual glucose | ... | ... | ... | ... | gm. | 1.780 | 1.802 |
| „ in CO ₂ in solution | ... | ... | ... | ... | „ | 0.016 | 0.038 |
| „ in volatile acids | ... | ... | ... | ... | „ | 0.024 | 0.016 |
| „ in non-volatile acids | ... | ... | ... | ... | „ | 0.223 | 0.182 |
| „ in volatile neutral compounds | ... | ... | ... | ... | „ | 0.001 | 0.003 |
| „ in synthetic compounds | ... | ... | ... | ... | „ | 0.137 | 0.152 |
| Total carbon accounted for | ... | ... | ... | ... | „ | 2.181 | 2.193 |
| „ „ in solution | ... | ... | ... | ... | „ | 2.303 | 2.439 |
| Carbon unaccounted for (by difference) | ... | ... | ... | ... | „ | 0.122 | 0.246 |
| <i>Residual Glucose.</i> | | | | | | | |
| Glucose (by polarimeter) | ... | ... | ... | ... | per cent. | 0.896 | 0.910 |
| „ (SHAFFER-HARTMANN) | ... | ... | ... | ... | „ | 0.890 | 0.901 |
| „ (WOOD-OST) | ... | ... | ... | ... | „ | 0.920 | 0.916 |
| „ (by alkaline iodine) | ... | ... | ... | ... | „ | 0.938 | 0.963 |
| <i>Acids.</i> | | | | | | Decrease of | Decrease of |
| Titration (N/1 acid) | ... | ... | ... | ... | c.c. | 0.1 | 0.7 |
| Volatile acids (N/1 acid) | ... | ... | ... | ... | „ | 2.58 | 1.19 |
| Barium salts (weight) | ... | ... | ... | ... | gm. | 0.110 | 0.066 |
| Calcium salts (weight) | ... | ... | ... | ... | „ | 0.899 | 0.994 |
| Volume of oxygen absorbed | ... | ... | ... | ... | c.c. | 2788 | 1797 |
| Respiration coefficient | ... | ... | ... | ... | ... | 1.16 | 1.28 |
| Mycelium (weight) | ... | ... | ... | ... | gm. | 1.526 | 2.001 |
| „ (carbon) | ... | ... | ... | ... | per cent. | 51.1 | 52.0 |

Genus 3. *Trichoderma*, 2 species.

- (1) *Trichoderma* species, Catalogue No. Ag. 47. Isolated at Ardeer from pale green spots on wood.
- (2) *Trichoderma lignorum* (TODE.) HARZ, Catalogue No. Ag. 22. Isolated at Ardeer from beech bark.

The carbon balance sheets for these two species are given in Table V.

Neither of the species of *Trichoderma* investigated grew well on CZAPEK-DOX solution, as judged by the weight of mycelium produced. They both give the same type of balance sheet, which indicates, in both cases, the production of considerable amounts of volatile neutral compounds. There are no other items of particular interest in either of the balance sheets.

Genus 4. *Cephalothecium*, 1 species.

- (1) *Cephalothecium roseum* CORDA, Catalogue No. Ag. 21. Isolated at Ardeer from a decaying twig.

The carbon balance sheet for this species, which is given in Table VI, shows no characteristics of any particular interest. This species grew well on CZAPEK-DOX solution, but was only able to metabolise very slowly the glucose supplied, since, even after 67 days, only about 65 per cent. of the glucose had been utilised. It apparently gives rise to no metabolic product other than carbon dioxide, in appreciable amounts.

TABLE V.—Carbon balance sheets for species of *Trichoderma*.

| Species of <i>Trichoderma</i> : | | | | | | <i>T. species.</i> | <i>T. lignorum.</i> |
|--|-----|-----|-----|-----|-----------|--------------------|---------------------|
| Catalogue number : | | | | | | Ag. 47 | Ag. 22 |
| Experiment number : | | | | | | C 4 | C 6 |
| Incubation period in days : | | | | | | 50 | 79 |
| <i>Carbon Balance Sheet.</i> | | | | | | | |
| Carbon in solution (start) | ... | ... | ... | ... | gm. | 5.043 | 5.043 |
| Carbon in H ₂ SO ₄ | ... | ... | ... | ... | " | 0.009 | 0.011 |
| „ in CO ₂ ... | ... | ... | ... | ... | " | 1.474 | 1.320 |
| „ in mycelium | ... | ... | ... | ... | " | 0.220 | 0.375 |
| „ in solution (end) | ... | ... | ... | ... | " | 3.086 | 3.141 |
| „ accounted for | ... | ... | ... | ... | " | 4.789 | 4.847 |
| „ accounted for | ... | ... | ... | ... | per cent. | 95.0 | 96.1 |
| <i>Analysis of Solution.</i> | | | | | | | |
| Carbon in residual glucose | ... | ... | ... | ... | gm. | 1.978 | 2.454 |
| „ in CO ₂ in solution | ... | ... | ... | ... | " | 0.008 | 0.006 |
| „ in volatile acids | ... | ... | ... | ... | " | 0.028 | 0.011 |
| „ in non-volatile acids | ... | ... | ... | ... | " | 0.098 | 0.118 |
| „ in volatile neutral compounds | ... | ... | ... | ... | " | 0.620 | 0.383 |
| „ in synthetic compounds | ... | ... | ... | ... | " | 0.147 | 0.104 |
| Total carbon accounted for | ... | ... | ... | ... | " | 2.879 | 3.076 |
| „ „ in solution | ... | ... | ... | ... | " | 3.086 | 3.141 |
| Carbon unaccounted for (by difference) | ... | ... | ... | ... | " | 0.207 | 0.065 |
| <i>Residual Glucose.</i> | | | | | | | |
| Glucose (by polarimeter) | ... | ... | ... | ... | per cent. | 1.090 | 1.209 |
| „ (SHAFFER-HARTMANN) | ... | ... | ... | ... | " | 0.989 | 1.227 |
| „ (WOOD-OST) | ... | ... | ... | ... | " | 1.028 | 1.212 |
| „ (by alkaline iodine) | ... | ... | ... | ... | " | 1.094 | 1.247 |
| <i>Acids.</i> | | | | | | | Decrease of |
| Titration (N/1 acid) | ... | ... | ... | ... | c.c. | 2.5 | 0.5 |
| Volatile acids (N/1 acid) | ... | ... | ... | ... | " | 1.77 | — |
| Barium salts (weight) | ... | ... | ... | ... | gm. | 0.131 | 0.042 |
| Calcium salts (weight) | ... | ... | ... | ... | " | 0.498 | 0.684 |
| Volume of oxygen absorbed | ... | ... | ... | ... | c.c. | 1984 | 2061 |
| Respiration coefficient | ... | ... | ... | ... | ... | 1.40 | 1.20 |
| Mycelium (weight) | ... | ... | ... | ... | gm. | 0.407 | 0.759 |
| „ (carbon) | ... | ... | ... | ... | per cent. | 54.1 | 49.4 |

TABLE VI.—Carbon balance sheet for a species of *Cephalothecium*.

| Species of <i>Cephalothecium</i> : | | | | | | <i>C. roseum</i> . |
|--|-----|-----|-----|-----|-----------|--------------------|
| Catalogue number : | | | | | | Ag. 21 |
| Experiment number : | | | | | | C 1 |
| Incubation period in days : | | | | | | 67 |
| <i>Carbon Balance Sheet.</i> | | | | | | |
| Carbon in solution (start) | ... | ... | ... | ... | gm. | 5.018 |
| Carbon in H ₂ SO ₄ ... | ... | ... | ... | ... | " | 0.001 |
| " in CO ₂ ... | ... | ... | ... | ... | " | 1.780 |
| " in mycelium | ... | ... | ... | ... | " | 0.853 |
| " in solution (end) | ... | ... | ... | ... | " | 2.287 |
| " accounted for | ... | ... | ... | ... | " | 4.921 |
| " accounted for | ... | ... | ... | ... | per cent. | 98.1 |
| <i>Analysis of Solution.</i> | | | | | | |
| Carbon in residual glucose | ... | ... | ... | ... | gm. | 1.774 |
| " in CO ₂ in solution | ... | ... | ... | ... | " | 0.049 |
| " in volatile acids | ... | ... | ... | ... | " | 0.053 |
| " in non-volatile acids | ... | ... | ... | ... | " | 0.175 |
| " in volatile neutral compounds | ... | ... | ... | ... | " | 0.077 |
| " in synthetic compounds | ... | ... | ... | ... | " | 0.147 |
| Total carbon accounted for | ... | ... | ... | ... | " | 2.275 |
| " in solution | ... | ... | ... | ... | " | 2.287 |
| Carbon unaccounted for (by difference) | ... | ... | ... | ... | " | 0.012 |
| <i>Residual Glucose.</i> | | | | | | |
| Glucose (by polarimeter) | ... | ... | ... | ... | per cent. | 0.918 |
| " (SHAFFER-HARTMANN) | ... | ... | ... | ... | " | 0.887 |
| " (WOOD-OST) | ... | ... | ... | ... | " | 0.868 |
| " (by alkaline iodine) | ... | ... | ... | ... | " | 0.875 |
| <i>Acids.</i> | | | | | | Decrease of |
| Titration (N/1 acid) | ... | ... | ... | ... | c.c. | 0.7 |
| Volatile acids (N/1 acid) | ... | ... | ... | ... | " | 2.20 |
| Barium salts (weight) | ... | ... | ... | ... | gm. | 0.269 |
| Calcium salts (weight) | ... | ... | ... | ... | " | 0.763 |
| Volume of oxygen absorbed | ... | ... | ... | ... | c.c. | 2867 |
| Respiration coefficient | ... | ... | ... | ... | ... | 1.19 |
| Mycelium (weight) | ... | ... | ... | ... | gm. | 1.624 |
| " (carbon) | ... | ... | ... | ... | per cent. | 52.5 |

FUNGI IMPERFECTI.

Order : HYPHOMYCETALES.

Family : DEMATIACEÆ.

Genus 1. *Cladosporium*, 5 species.

- (1) *Cladosporium* species S, Catalogue No. Ag. 1. Isolated from cold storage meat by Mr. F. T. BROOKS, of Cambridge.
- (2) *Cladosporium* species 1020, Catalogue No. Ag. 3. Isolated from cold storage meat by Mr. F. T. BROOKS.
- (3) *Cladosporium* species 60, Catalogue No. Ag. 5. Isolated from dead leaves by Mr. F. T. BROOKS.
- (4) *Cladosporium* species 55, Catalogue No. Ag. 9. Isolated from a maize shoot by Mr. F. T. BROOKS.
- (5) *Cladosporium* species from lichen, Catalogue No. Ag. 109. Isolated at Ardeer from a lichen.

The carbon balance sheets for these five species are given in Table VII.

All the species of *Cladosporium* grew quite reasonably well on CZAPEK-DOX solution, but with one exception did not metabolize very quickly the glucose provided. The single exception is *Cladosporium* species 55, which completely destroyed all the glucose in 76 days. From a biochemical point of view, the balance sheets for these five species, which are all of the same type, are very uninteresting, since no compounds of any sort other than carbon dioxide are produced in an appreciable amount. None of these species produced appreciable amounts of acid, either of a volatile or non-volatile nature, and in three cases out of five there is an actual decrease in the titratable acidity of the medium. Of minor interest is the uniformly high percentage of carbon in the mycelium.

Genus 2. *Helminthosporium*, 6 species.

- (1) *Helminthosporium geniculatum* TRACY et EARLE, Catalogue No. Ag. 93.
- (2) *Helminthosporium teres* SACC., Catalogue No. Ag. 94.
- (3) *Helminthosporium inaequalis* SHEAR., Catalogue No. Ag. 95.
- (4) *Helminthosporium* species, Catalogue No. Ag. 96.
- (5) *Helminthosporium interseminatum* BERK. et RAV., Catalogue No. Ag. 97.
- (6) *Helminthosporium gramineum* RABENH., Catalogue No. Ag. 98.

All these cultures were purchased from the Centraalbureau voor Schimmelcultures at Baarn.

The carbon balance sheets for these six species, which are given in Table VIII, are all of a similar type, and enable one to classify biochemically, species of *Helminthosporium*

TABLE VII.—Carbon balance sheets for species of *Cladosporium*.

| Species of <i>Cladosporium</i> : | Species S. | Species 1020 | Species 60 | Species 55 | Species fr. lichen. |
|---|------------|--------------|------------|------------|---------------------|
| Catalogue number : | Ag. 1 | Ag. 3 | Ag. 5 | Ag. 9 | Ag. 109 |
| Experiment number : | B 4 | B 5 | B 7 | B 9 | B 23 |
| Incubation period in days : | 81 | 72 | 52 | 76 | 50 |
| <i>Carbon Balance Sheet.</i> | | | | | |
| Carbon in solution (start) gm. | 4·901 | 5·043 | 5·043 | 5·043 | 4·952 |
| Carbon in H ₂ SO ₄ " | Nil | Nil | Nil | 0·004 | Nil |
| " in CO ₂ " | 1·503 | 1·230 | 1·451 | 2·524 | 0·892 |
| " in mycelium " | 1·282 | 0·909 | 1·260 | 2·025 | 0·521 |
| " in solution (end) " | 2·033 | 2·680 | 2·115 | 0·181 | 3·499 |
| " accounted for " | 4·818 | 4·819 | 4·826 | 4·734 | 4·912 |
| " accounted for per cent. | 98·3 | 96·0 | 96·2 | 94·4 | 99·2 |
| <i>Analysis of Solution.</i> | | | | | |
| Carbon in residual glucose gm. | 1·684 | 2·432 | 1·620 | 0·021 | 3·186 |
| " in CO ₂ in solution " | 0·019 | 0·015 | 0·016 | 0·028 | 0·009 |
| " in volatile acids " | 0·009 | 0·007 | 0·060 | 0·002 | 0·009 |
| " in non-volatile acids " | 0·080 | 0·066 | 0·103 | 0·103 | 0·190 |
| " in volatile neutral compounds " | Nil | 0·001 | 0·007 | Nil | 0·006 |
| " in synthetic compounds " | 0·051 | 0·040 | 0·049 | 0·006 | 0·101 |
| Total carbon accounted for " | 1·843 | 2·561 | 1·855 | 0·160 | 3·501 |
| " " in solution " | 2·033 | 2·680 | 2·115 | 0·181 | 3·499 |
| Carbon unaccounted for (by difference) " | 0·190 | 0·119 | 0·260 | 0·021 | Surplus of 0·002 |
| <i>Residual Glucose.</i> | | | | | |
| Glucose (by polarimeter) per cent. | 0·934 | 1·318 | 0·874 | 0·018 | 1·354 |
| " (SHAFFER-HARTMANN) " | 0·842 | 1·216 | 0·810 | 0·011 | 1·593 |
| " (WOOD-OST) " | 0·880 | 1·222 | 0·824 | — | 1·374 |
| " (by alkaline iodine) " | 0·863 | 1·277 | 0·859 | 0·019 | 1·598 |
| <i>Acids.</i> | | | | | |
| Titration (N/1 acid) c.c. | 0·1 | 0·1 | 0·3 | 1·4 | 0·2 |
| Volatile acids (N/1 acid) " | 1·05 | 0·86 | 1·91 | 0·18 | 0·35 |
| Barium salts (weight) gm. | 0·069 | 0·036 | 0·294 | 0·017 | 0·075 |
| Calcium salts (weight) " | 0·517 | 0·396 | 0·585 | 0·804 | 0·728 |
| Volume of oxygen absorbed c.c. | 2281 | 1905 | 2148 | 3988 | 1524 |
| Respiration coefficient " | 1·25 | 1·22 | 1·28 | 1·20 | 1·10 |
| Mycelium (weight) gm. | 2·341 | 1·663 | 2·230 | 3·600 | 0·974 |
| " (carbon) per cent. | 54·7 | 54·8 | 56·5 | 56·3 | 53·5 |

TABLE VIII.—Carbon balance sheets for species of *Helminthosporium*.

| Species of <i>Helminthosporium</i> : | <i>H. geniculatum</i> . | <i>H. teres</i> . | <i>H. inaequalis</i> . | <i>H. species</i> . | <i>H. interseminatum</i> . | <i>H. gramineum</i> . |
|---|-------------------------|-------------------|------------------------|---------------------|----------------------------|-----------------------|
| Catalogue number : | Ag. 93 | Ag. 94 | Ag. 95 | Ag. 96 | Ag. 97 | Ag. 98 |
| Experiment number : | B 15 | B 16 | B 17 | B 18 | B 19 | B 20 |
| Incubation period in days : | 53 | 65 | 61 | 63 | 66 | 37 |
| <i>Carbon Balance Sheet.</i> | | | | | | |
| Carbon in solution (start) ... gm. | 4.952 | 4.952 | 4.952 | 4.952 | 4.952 | 4.952 |
| Carbon in H ₂ SO ₄ " | 0.015 | 0.003 | 0.005 | 0.002 | 0.001 | 0.003 |
| „ in CO ₂ " | 2.017 | 1.547 | 2.062 | — | 1.763 | 1.350 |
| „ in mycelium " | 0.906 | 0.700 | 1.460 | 0.526 | 0.630 | 0.659 |
| „ in solution (end) " | 1.827 | 2.473 | 1.242 | 2.706 | 2.489 | 2.783 |
| „ accounted for " | 4.765 | 4.723 | 4.769 | — | 4.883 | 4.795 |
| „ accounted for per cent. | 96.2 | 95.4 | 96.3 | — | 98.8 | 96.8 |
| <i>Analysis of Solution.</i> | | | | | | |
| Carbon in residual glucose... gm. | 0.242 | 2.206 | 0.964 | 2.350 | 2.214 | 2.384 |
| „ in CO ₂ in solution .. " | 0.007 | 0.002 | 0.008 | 0.005 | 0.012 | 0.032 |
| „ in volatile acids " | 0.029 | 0.013 | 0.013 | 0.013 | 0.055 | 0.048 |
| „ in non-volatile acids .. " | 0.094 | 0.077 | 0.109 | 0.080 | 0.148 | 0.078 |
| „ in volatile neutral compounds " | 0.818 | 0.141 | 0.048 | 0.107 | 0.042 | 0.258 |
| „ in synthetic compounds,, | 0.015 | 0.020 | 0.015 | 0.026 | 0.058 | 0.043 |
| Total carbon accounted for .. | 1.205 | 2.459 | 1.157 | 2.581 | 2.529 | 2.843 |
| „ „ in solution " | 1.827 | 2.473 | 1.242 | 2.706 | 2.489 | 2.783 |
| Carbon unaccounted for (by difference) .. | 0.622 | 0.014 | 0.085 | 0.125 | Surplus of 0.040 | Surplus of 0.060 |
| <i>Residual Glucose.</i> | | | | | | |
| Glucose (by polarimeter) per cent. | 0.100 | 1.072 | 0.495 | 1.120 | 0.822 | 1.135 |
| „ (SHAFFER-HARTMANN) per cent. | 0.121 | 1.103 | 0.483 | 1.175 | 1.107 | 1.192 |
| „ (WOOD-OST) .. " | — | 0.954 | — | 1.134 | 0.968 | 1.126 |
| „ (by alkaline iodine) .. " | 0.162 | 1.106 | 0.493 | 1.189 | 1.060 | 1.167 |
| <i>Acids.</i> | | | | | | |
| Titration (N/1 acid) ... c.c. | 0.3 | Decrease of 1.1 | Decrease of 0.8 | Decrease of 1.2 | 0.3 | Decrease of 0.7 |
| Volatile acids (N/1 acid) " | 1.44 | — | 0.87 | 0.68 | 1.74 | 1.99 |
| Barium salts (weight) ... gm. | 0.154 | 0.063 | 0.057 | 0.049 | 0.237 | 0.229 |
| Calcium salts (weight) " | 0.449 | 0.413 | 0.571 | 0.510 | 0.548 | 0.783 |
| Volume of oxygen absorbed c.c. | 2417 | 2554 | 3351 | 2746 | 3106 | 1974 |
| Respiration coefficient " | 1.56 | 1.13 | 1.16 | — | 1.07 | 1.31 |
| Mycelium (weight) gm. | 1.577 | 1.362 | 2.826 | 1.008 | 1.148 | 1.285 |
| „ (carbon) per cent. | 57.4 | 51.4 | 51.7 | 52.2 | 53.7 | 51.3 |

with those fungi which produce moderate amounts of volatile neutral compounds (alcohol). All the species grow reasonably well on CZAPEK-DOX solution. None of the species gives rise to appreciable amounts of either carbon as non-volatile acids or as titratable acidity, and in fact, four of the species actually produce a decrease in the initial acidity of the medium. Five out of six species give negligible, or even negative amounts of "carbon unaccounted for," but the other species, *Helminthosporium geniculatum*, provides the first instance so far recorded, in this paper, of any of the miscellaneous fungi worthy of further intensive investigation. This species has a figure for "carbon unaccounted for" of 0.622 gm., corresponding to a yield of 13 per cent. of the glucose fermented. This, together with 0.818 gm. of "carbon as volatile neutral compounds," corresponding to a yield of 17 per cent., gives a total yield of metabolic products other than carbon dioxide of 30 per cent. An investigation has been carried out on the nature of the compounds included in the "carbon unaccounted for" produced by this species, and is reported in Part XVII.

Genus 3. *Heterosporium*, 2 species.

- (1) *Heterosporium gracile* SACC., Catalogue No. Ag. 99. Purchased from Baarn.
- (2) *Heterosporium variabile* COOKE, Catalogue No. Ag. 100. Purchased from Baarn.

The carbon balance sheets for these two species are given in Table IX. Both species grow well on the CZAPEK-DOX medium, and both give balance sheets of a similar type, *i.e.*, the type producing no volatile neutral compounds. Ag. 100 might, if occasion offered, prove a suitable species for further investigation, since it gives a figure for "carbon unaccounted for" of 0.381 gm. (11 per cent.), and a figure for carbon in non-volatile acids of 0.228 gm. (7 per cent.).

Genus 4. *Alternaria*, 3 species.

- (1) *Alternaria* species, Catalogue No. Ag. 35. Isolated at Ardeer from tobacco leaf spots.
- (2) *Alternaria* species, Catalogue No. Ag. 16. Isolated at Ardeer from black spots on butter.
- (3) *Alternaria* species, Catalogue No. Ag. 49. Isolated at Ardeer from a rotting orange.

The carbon balance sheets for these three species are given in Table X, and are all of the same type. The main metabolic product is that included in "volatile neutral compounds," and while only moderate in amount (limits 0.262 gm. to 0.171 gm.) it is perfectly definite. These three species also have respiration coefficients corresponding to this feature (limits 1.31 to 1.26), but do not give rise to any other metabolic products except a small amount of some compound included in "carbon unaccounted for" (limits 0.285 gm. to 0.209 gm.).

TABLE IX.—Carbon balance sheets for species of *Heterosporium*.

| Species of <i>Heterosporium</i> : | | | | | | <i>H. gracile</i> . | <i>H. variable</i> . |
|--|-----|-----|-----|-----|-----------|---------------------|----------------------|
| Catalogue number : | | | | | | Ag. 99 | Ag. 100 |
| Experiment number : | | | | | | B 21 | B 22 |
| Incubation period in days : | | | | | | 68 | 44 |
| <i>Carbon Balance Sheet.</i> | | | | | | | |
| Carbon in solution (start) | ... | ... | ... | ... | gm. | 4.952 | 4.952 |
| Carbon in H ₂ SO ₄ | ... | ... | ... | ... | ... | 0.001 | 0.001 |
| " in CO ₂ ... | ... | ... | ... | ... | ... | 2.256 | 1.545 |
| " in mycelium | ... | ... | ... | ... | ... | 1.155 | 1.031 |
| " in solution (end) | ... | ... | ... | ... | ... | 1.394 | 2.268 |
| " accounted for | ... | ... | ... | ... | ... | 4.806 | 4.845 |
| " accounted for | ... | ... | ... | ... | per cent. | 97.3 | 97.8 |
| <i>Analysis of Solution.</i> | | | | | | | |
| Carbon in residual glucose | ... | ... | ... | ... | gm. | 0.975 | 1.585 |
| " in CO ₂ in solution | ... | ... | ... | ... | ... | 0.016 | 0.004 |
| " in volatile acids | ... | ... | ... | ... | ... | 0.041 | 0.021 |
| " in non-volatile acids | ... | ... | ... | ... | ... | 0.134 | 0.228 |
| " in volatile neutral compounds | ... | ... | ... | ... | ... | Nil | 0.002 |
| " in synthetic compounds | ... | ... | ... | ... | ... | 0.071 | 0.047 |
| Total carbon accounted for | ... | ... | ... | ... | ... | 1.237 | 1.887 |
| " " in solution | ... | ... | ... | ... | ... | 1.394 | 2.268 |
| Carbon unaccounted for (by difference) | ... | ... | ... | ... | ... | 0.157 | 0.381 |
| <i>Residual Glucose.</i> | | | | | | | |
| Glucose (by polarimeter) | ... | ... | ... | ... | per cent. | 0.340 | 0.889 |
| " (SHAFFER-HARTMANN) | ... | ... | ... | ... | ... | 0.488 | 0.792 |
| " (WOOD-OST) | ... | ... | ... | ... | ... | 0.438 | 0.726 |
| " (by alkaline iodine) | ... | ... | ... | ... | ... | 0.445 | 0.831 |
| <i>Acids.</i> | | | | | | Decrease of | |
| Titration (N/1 acid) | ... | ... | ... | ... | c.c. | 0.6 | 0.9 |
| Volatile acids (N/1 acid) | ... | ... | ... | ... | ... | 3.95 | 1.26 |
| Barium salts (weight) | ... | ... | ... | ... | gm. | 0.135 | 0.097 |
| Calcium salts (weight) | ... | ... | ... | ... | ... | 0.431 | 0.882 |
| Volume of oxygen absorbed | ... | ... | ... | ... | c.c. | 3893 | 2465 |
| Respiration coefficient | ... | ... | ... | ... | ... | 1.09 | 1.17 |
| Mycelium (weight) | ... | ... | ... | ... | gm. | 2.330 | 2.036 |
| " (carbon) | ... | ... | ... | ... | per cent. | 49.6 | 50.6 |

TABLE X.—Carbon balance sheets for species of *Alternaria*.

| Species of <i>Alternaria</i> : | | | | | <i>A. species.</i> | <i>A. species.</i> | <i>A. species.</i> |
|---|--|--|--|--|--------------------|--------------------|--------------------|
| Catalogue number : | | | | | Ag. 35 | Ag. 16 | Ag. 49 |
| Experiment number : | | | | | B 6 | B 11 | B 12 |
| Incubation period in days : | | | | | 67 | 44 | 64 |
| <i>Carbon Balance Sheet.</i> | | | | | | | |
| Carbon in solution (start) gm. | | | | | 5.043 | 5.043 | 5.043 |
| Carbon in H ₂ SO ₄ " | | | | | 0.006 | 0.006 | 0.005 |
| " in CO ₂ " | | | | | 1.188 | 1.816 | 2.346 |
| " in mycelium " | | | | | 0.433 | 1.064 | 1.193 |
| " in solution (end) " | | | | | 3.210 | 1.998 | 1.266 |
| " accounted for " | | | | | 4.837 | 4.884 | 4.810 |
| " accounted for per cent. | | | | | 95.9 | 96.9 | 95.4 |
| <i>Analysis of Solution.</i> | | | | | | | |
| Carbon in residual glucose gm. | | | | | 2.494 | 1.300 | 0.527 |
| " in CO ₂ in solution " | | | | | 0.011 | 0.015 | 0.030 |
| " in volatile acids " | | | | | 0.024 | 0.098 | 0.021 |
| " in non-volatile acids " | | | | | 0.091 | 0.072 | 0.145 |
| " in volatile neutral compounds " | | | | | 0.262 | 0.261 | 0.171 |
| " in synthetic compounds " | | | | | 0.059 | 0.043 | 0.087 |
| Total carbon accounted for " | | | | | 2.941 | 1.789 | 0.981 |
| " " in solution " | | | | | 3.210 | 1.998 | 1.266 |
| Carbon unaccounted for (by difference) " | | | | | 0.269 | 0.209 | 0.285 |
| <i>Residual Glucose.</i> | | | | | | | |
| Glucose (by polarimeter) per cent. | | | | | 1.426 | 0.684 | 0.226 |
| " (SHAFFER-HARTMANN) " | | | | | 1.247 | 0.650 | 0.263 |
| " (WOOD-OST) " | | | | | 1.254 | 0.648 | — |
| " (by alkaline iodine) " | | | | | 1.342 | 0.692 | 0.319 |
| <i>Acids.</i> | | | | | | | |
| Titration (N/1 acid) c.c. | | | | | Nil | 1.0 | Decrease of 0.8 |
| Volatile acids (N/1 acid) " | | | | | 1.28 | 4.37 | 0.74 |
| Barium salts (weight) gm. | | | | | 0.073 | 0.517 | 0.077 |
| Calcium salts (weight) " | | | | | 0.615 | 0.354 | 0.797 |
| Volume of oxygen absorbed c.c. | | | | | 1704 | 2668 | 3534 |
| Respiration coefficient " | | | | | 1.31 | 1.28 | 1.26 |
| Mycelium (weight) gm. | | | | | 0.849 | 1.999 | 2.237 |
| " (carbon) per cent. | | | | | 51.0 | 53.2 | 53.3 |

Genus 5. *Fumago*, 2 species.

- (1) *Fumago* species, Catalogue No. Ag. 63. Isolated at Ardeer as a bench contaminant of beer wort.
- (2) *Fumago vagans* PERS., Catalogue No. Ag. 92. Purchased from Baarn.

The carbon balance sheets for these two species are given in Table XI. In the case of *Fumago vagans*, Ag. 92, two balance sheets are given. The first one, B 14, was prepared from a culture grown on the usual CZAPEK-DOX solution containing 0.2 per cent. sodium nitrate as the source of nitrogen. The second balance sheet for this species, B 14 (X), was prepared from a culture grown on a similar CZAPEK-DOX solution in which, however, 0.2 per cent. ammonium nitrate was used as the source of nitrogen in place of the usual 0.2 per cent. sodium nitrate.

Both species of *Fumago* give balance sheets which are decidedly interesting from a biochemical point of view, although they are slightly different in type, because of the fact that, while Ag. 63 gives appreciable amounts of volatile neutral compounds (0.320 gm.), Ag. 92 gives only a negligible amount of the same type of compound (0.010 gm.). Of particular interest is the large amount of "carbon unaccounted for" given by both species and amounting to 0.512 gm. (15 per cent. of the glucose fermented) for Ag. 63, 0.965 gm. (19.8 per cent.) for Ag. 92 on sodium nitrate, and 0.468 gm. (19.5 per cent.) for Ag. 92 on ammonium nitrate. Another interesting feature is the relatively high titratable acidity and "carbon in non-volatile acids," which amounts to 0.189 gm. (5.4 per cent.) for Ag. 63, 0.407 gm. (8.4 per cent.) for Ag. 92 on sodium nitrate, and 0.268 gm. (11 per cent.) for Ag. 92 on ammonium nitrate. There is also a very marked difference in the percentage of glucose estimated by the polarimeter and SHAFFER-HARTMANN methods, for, with Ag. 92 on sodium nitrate, the respective figures are 0.344 and 0.043 per cent., while with the same species on ammonium nitrate the figures are 1.709 and 1.300 per cent. It is thus obvious that this species produces some material from glucose which is optically dextro-rotatory, but which has no reducing effect on alkaline copper solutions.

Both species of *Fumago*, and particularly *Fumago vagans*, Ag. 92, are thus marked out as species worthy of further investigation. An account of the metabolic products isolated from *Fumago vagans*, Ag. 92, is given in Part XVII.

Genus 6. *Clasterosporium*, 2 species.

- (1) *Clasterosporium* species, Catalogue No. Ag. 64. Isolated at Ardeer from rotting cotton pulp at the methane plant. Identified by Mr. F. T. BROOKS, of Cambridge.
- (2) *Clasterosporium* species, Catalogue No. Ag. 15. Isolated by Mr. F. T. BROOKS from sea-weed.

TABLE XI.—Carbon balance sheets for species of *Fumago*.

| Species of <i>Fumago</i> : | <i>Fumago</i> species. | <i>Fumago</i> <i>vagans</i> . | <i>Fumago</i> <i>vagans</i> on NH_4NO_3 . |
|---|---------------------------|----------------------------------|---|
| Catalogue number : | Ag. 63 | Ag. 92 | Ag. 92 |
| Experiment number : | B 3 | B 14 | B 14 (X) |
| Incubation period in days : | 77 | 75 | 71 |
| <i>Carbon Balance Sheet.</i> | | | |
| Carbon in solution (start) gm. | 5.018 | 4.952 | 4.975 |
| Carbon in H_2SO_4 " | 0.003 | 0.001 | 0.001 |
| " in CO_2 " | 1.472 | 1.860 | 0.751 |
| " in mycelium " | 0.651 | 1.440 | 0.617 |
| " in solution (end) " | 2.780 | 1.582 | 3.544 |
| " accounted for " | 4.906 | 4.883 | 4.913 |
| " accounted for per cent. | 97.8 | 98.6 | 97.9 |
| <i>Analysis of Solution.</i> | | | |
| Carbon in residual glucose gm. | 1.538 | 0.087 | 2.600 |
| " in CO_2 in solution " | 0.008 | Nil | Nil |
| " in volatile acids " | 0.049 | 0.004 | 0.047 |
| " in non-volatile acids " | 0.189 | 0.407 | 0.268 |
| " in volatile neutral compounds " | 0.320 | 0.010 | 0.023 |
| " in synthetic compounds " | 0.164 | 0.109 | 0.138 |
| Total carbon accounted for " | 2.268 | 0.617 | 3.076 |
| " " in solution " | 2.780 | 1.582 | 3.544 |
| Carbon unaccounted for (by difference) " | 0.512 | 0.965 | 0.468 |
| <i>Residual Glucose.</i> | | | |
| Glucose (by polarimeter) per cent. | 0.794 | 0.344 | 1.709 |
| " (SHAFFER-HARTMANN) " | 0.769 | 0.043 | 1.300 |
| " (WOOD-OST) " | 0.762 | — | 1.370 |
| " (by alkaline iodine) " | 0.835 | 0.117 | 1.395 |
| <i>Acids.</i> | | | |
| Titration (N/1 acid) c.c. | 2.3 | 7.0 | 7.6 |
| Volatile acids (N/1 acid) " | 3.20 | — | 2.13 |
| Barium salts (weight) gm. | 0.262 | 0.016 | 0.260 |
| Calcium salts (weight) " | 0.981 | 1.363 | 1.029 |
| Volume of oxygen absorbed c.c. | 2177 | 3144 | 1220 |
| Respiration coefficient " | 1.27 | 1.11 | 1.15 |
| Mycelium (weight) gm. | 1.224 | 2.917 | 1.218 |
| " (carbon) per cent. | 53.2 | 49.4 | 50.7 |

The carbon balance sheets for these two species are given in Table XII. They are both biochemically interesting, although they differ in type, for while Ag. 64 gives a very considerable amount of volatile neutral compounds, 0.690 gm. (14 per cent. of glucose fermented), Ag. 15 gives only a negligible amount of the same type of product, 0.006 gm. (0.3 per cent.). Both of them, however, give very appreciable amounts of products in the class "carbon unaccounted for." Under this heading Ag. 64 gives 1.070 gm. of carbon (22 per cent.), while Ag. 15 gives 0.356 gm. (9 per cent.). Neither species shows any other item of biochemical interest. It is obvious that both these species, and particularly *Clasterosporium* species, Ag. 64, are worthy of further investigation, and an account of an investigation of the metabolic products of *Clasterosporium*, species Ag. 64, is given in Part XVII.

Genus 7. *Rhacodium*, 1 species.

- (1) *Rhacodium cellare* PERS., Catalogue No. Ag. 13. Isolated at Ardeer from wine cellar debris.

The carbon balance sheet for this species is given in Table XIII.

The only point of biochemical interest about this carbon balance sheet is the fact that *Rhacodium cellare* appears to form small amounts, 0.158 gm. (6.3 per cent.), of a non-volatile acid which may possibly be optically dextro-rotatory, since there is a considerable difference between the glucose as estimated by the polarimeter (1.512 per cent.) and by the SHAFFER-HARTMANN method (1.257 per cent.). With the exception of this there is a complete absence of any other features of biochemical interest.

TABLE XII.—Carbon balance sheets for species of *Clasterosporium*.

| Species of <i>Clasterosporium</i> : | | | | | | C. species. | C. species. |
|--|-----|-----|-----|-----|-----------|-------------|-------------|
| Catalogue number : | | | | | | Ag. 64 | Ag. 15 |
| Experiment number : | | | | | | B 8 | B 10 |
| Incubation period in days : | | | | | | 39 | 66 |
| <i>Carbon Balance Sheet.</i> | | | | | | | |
| Carbon in solution (start) | ... | ... | ... | ... | gm. | 5.043 | 5.043 |
| Carbon in H ₂ SO ₄ | ... | ... | ... | ... | .. | 0.008 | 0.001 |
| " in CO ₂ ... | ... | ... | ... | ... | .. | 1.723 | 1.945 |
| " in mycelium | ... | ... | ... | ... | .. | 0.857 | 1.302 |
| " in solution (end) | ... | ... | ... | ... | .. | 2.237 | 1.535 |
| " accounted for | ... | ... | ... | ... | .. | 4.825 | 4.783 |
| " accounted for | ... | ... | ... | ... | per cent. | 95.7 | 94.9 |
| <i>Analysis of Solution.</i> | | | | | | | |
| Carbon in residual glucose | ... | ... | ... | ... | gm. | 0.176 | 0.928 |
| " in CO ₂ in solution | ... | ... | ... | ... | .. | 0.019 | 0.043 |
| " in volatile acids | ... | ... | ... | ... | .. | 0.025 | 0.019 |
| " in non-volatile acids | ... | ... | ... | ... | .. | 0.117 | 0.143 |
| " in volatile neutral compounds | ... | ... | ... | ... | .. | 0.690 | 0.006 |
| " in synthetic compounds | ... | ... | ... | ... | .. | 0.140 | 0.040 |
| Total carbon accounted for | ... | ... | ... | ... | .. | 1.167 | 1.179 |
| " " in solution | ... | ... | ... | ... | .. | 2.237 | 1.535 |
| Carbon unaccounted for (by difference) | ... | ... | ... | ... | .. | 1.070 | 0.356 |
| <i>Residual glucose.</i> | | | | | | | |
| Glucose (by polarimeter) | ... | ... | ... | ... | per cent. | 0.067 | 0.468 |
| " (SHAFFER-HARTMANN) | ... | ... | ... | ... | .. | 0.088 | 0.464 |
| " (WOOD-OST) | ... | ... | ... | ... | .. | — | 0.470 |
| " (by alkaline iodine) | ... | ... | ... | ... | .. | 0.158 | 0.481 |
| <i>Acids.</i> | | | | | | | Decrease of |
| Titration (N/1 acid) | ... | ... | ... | ... | c.c. | 0.4 | 0.9 |
| Volatile acids (N/1 acid) | ... | ... | ... | ... | .. | 1.05 | 0.69 |
| Barium salts (weight) | ... | ... | ... | ... | gm. | 0.100 | 0.083 |
| Calcium salts (weight) | ... | ... | ... | ... | .. | 0.624 | 0.808 |
| Volume of oxygen absorbed | ... | ... | ... | ... | c.c. | 1869 | 2846 |
| Respiration coefficient | ... | ... | ... | ... | ... | 1.74 | 1.30 |
| Mycelium (weight) | ... | ... | ... | ... | gm. | 1.608 | 2.338 |
| " (carbon) | ... | ... | ... | ... | per cent. | 53.3 | 55.6 |

TABLE XIII.—Carbon balance sheet for a species of *Rhacodium*.

| | | | | | |
|--|-----|-----|-----|-----------|----------------------------|
| Species of <i>Rhacodium</i> : | | | | | <i>Rhacodium cellare</i> . |
| Catalogue number : | | | | | Ag. 13 |
| Experiment number : | | | | | B 1 |
| Incubation period in days : | | | | | 60 |
| <i>Carbon Balance Sheet.</i> | | | | | |
| Carbon in solution (start) | ... | ... | ... | ... | gm. 5.018 |
| Carbon in H ₂ SO ₄ ... | ... | ... | ... | ... | gm. 0.001 |
| " in CO ₂ ... | ... | ... | ... | ... | gm. 1.048 |
| " in mycelium ... | ... | ... | ... | ... | gm. 0.995 |
| " in solution (end) | ... | ... | ... | ... | gm. 2.865 |
| " accounted for | ... | ... | ... | ... | gm. 4.909 |
| " accounted for | ... | ... | ... | per cent. | 97.8 |
| <i>Analysis of Solution.</i> | | | | | |
| Carbon in residual glucose | ... | ... | ... | ... | gm. 2.514 |
| " in CO ₂ in solution | ... | ... | ... | ... | gm. 0.004 |
| " in volatile acids | ... | ... | ... | ... | gm. 0.002 |
| " in non-volatile acids | ... | ... | ... | ... | gm. 0.158 |
| " in volatile neutral compounds | ... | ... | ... | ... | gm. 0.007 |
| " in synthetic compounds | ... | ... | ... | ... | gm. 0.071 |
| Total carbon accounted for | ... | ... | ... | ... | gm. 2.756 |
| " in solution | ... | ... | ... | ... | gm. 2.865 |
| Carbon unaccounted for (by difference) | ... | ... | ... | ... | gm. 0.109 |
| <i>Residual Glucose.</i> | | | | | |
| Glucose (by polarimeter) | ... | ... | ... | per cent. | 1.512 |
| " (SHAFFER-HARTMANN) | ... | ... | ... | " | 1.257 |
| " (WOOD-OST) | ... | ... | ... | " | 1.230 |
| " (by alkaline iodine) | ... | ... | ... | " | 1.267 |
| <i>Acids.</i> | | | | | Decrease of |
| Titration (N/1 acid) | ... | ... | ... | c.c. | 0.4 |
| Volatile acids (N/1 acid) | ... | ... | ... | " | 0.35 |
| Barium salts (weight) | ... | ... | ... | gm. | 0.029 |
| Calcium salts (weight) | ... | ... | ... | " | 0.571 |
| Volume of oxygen absorbed | ... | ... | ... | c.c. | 1914 |
| Respiration coefficient | ... | ... | ... | ... | 1.03 |
| Mycelium (weight) | ... | ... | ... | gm. | 1.972 |
| " (carbon) | ... | ... | ... | per cent. | 50.4 |

FUNGI IMPERFECTI.

Order : HYPHOMYCETALES.

Family : STILBACEÆ.

Genus 1. *Stysanus*, 1 species.

- (1) *Stysanus* species, Catalogue No. Ag. 51. Isolated at Ardeer from rotting pulped cotton.

The carbon balance sheet for this species, which is given in Table XIV, shows one item of outstanding biochemical interest. This is the figure for "carbon unaccounted for," 0.520 gm. (16.7 per cent. of the glucose fermented). It is obvious that this species should be further investigated with a view to elucidating the nature of the products included under this heading.

There are no other items of biochemical interest in this particular balance sheet.

FUNGI IMPERFECTI.

Order : HYPHOMYCETALES.

Family : TUBERCULARIACEÆ.

Genus 1. *Fusarium* (see Part V).Genus 2. *Epicoccum*, 2 species.

- (1) *Epicoccum* species, Catalogue No. Ag. 57. Isolated at Ardeer as a bench contaminant of CZAPEK-DOX agar.
- (2) *Epicoccum* species, Catalogue No. Ag. 32. Isolated at Ardeer from the grain of an infected wheat ear.

The carbon balance sheets for these two species of *Epicoccum* are given in Table XV. They are both very similar in character to the carbon balance sheets given by a certain type of *Fusarium*. Both species give appreciable amounts of volatile neutral compounds, and Ag. 57, at any rate, has a relatively high respiration coefficient, 1.64. They are most closely related to the type of *Fusarium* species given in Table I of Part V in the group including *Fusarium solani* and *Fusarium lini*. The characteristics which these *Epicoccum* species have in common with this group of *Fusaria*—and this applies more particularly to *Epicoccum* species, Ag. 32, than to the species Ag. 57—are (1) a moderate amount of "carbon unaccounted for" and (2) a relatively high value for "carbon in volatile acids." Thus the carbon in volatile acids for *Epicoccum* species, Ag. 32, which is 0.108 gm., is, while small in itself, very much larger than any other figure for the same type of compound given by any other species of fungus described in this paper.

TABLE XIV.—Carbon balance sheet for a species of *Stysanus*.

| Species of <i>Stysanus</i> : | | | | | | <i>Stysanus</i> species. |
|--|-----|-----|-----|-----|-----------|--------------------------|
| Catalogue number : | | | | | | Ag. 51 |
| Experiment number : | | | | | | B 13 |
| Incubation period in days : | | | | | | 60 |
| <i>Carbon Balance Sheet.</i> | | | | | | |
| Carbon in solution (start) | ... | ... | ... | ... | gm. | 4.952 |
| Carbon in H ₂ SO ₄ ... | ... | ... | ... | ... | " | 0.002 |
| " in CO ₂ ... | ... | ... | ... | ... | " | 1.530 |
| " in mycelium ... | ... | ... | ... | ... | " | 0.685 |
| " in solution (end) | ... | ... | ... | ... | " | 2.676 |
| " accounted for | ... | ... | ... | ... | " | 4.893 |
| " accounted for | ... | ... | ... | ... | per cent. | 96.6 |
| <i>Analysis of Solution.</i> | | | | | | |
| Carbon in residual glucose | ... | ... | ... | ... | gm. | 1.842 |
| " in CO ₂ in solution | ... | ... | ... | ... | " | 0.007 |
| " in volatile acids | ... | ... | ... | ... | " | 0.062 |
| " in non-volatile acids | ... | ... | ... | ... | " | 0.132 |
| " in volatile neutral compounds | ... | ... | ... | ... | " | 0.079 |
| " in synthetic compounds | ... | ... | ... | ... | " | 0.034 |
| Total carbon accounted for | ... | ... | ... | ... | " | 2.156 |
| " " in solution | ... | ... | ... | ... | " | 2.676 |
| Carbon unaccounted for (by difference) | ... | ... | ... | ... | " | 0.520 |
| <i>Residual Glucose.</i> | | | | | | |
| Glucose (by polarimeter) | ... | ... | ... | ... | per cent. | 0.927 |
| " (SHAFFER-HARTMANN) | ... | ... | ... | ... | " | 0.921 |
| " (WOOD-OST) | ... | ... | ... | ... | " | 0.924 |
| " (by alkaline iodine) | ... | ... | ... | ... | " | 1.023 |
| <i>Acids.</i> | | | | | | Decrease of |
| Titration (N/1 acid) | ... | ... | ... | ... | c.c. | 0.3 |
| Volatile acids (N/1 acid) | ... | ... | ... | ... | " | 0.23 |
| Barium salts (weight) | ... | ... | ... | ... | gm. | 0.032 |
| Calcium salts (weight) | ... | ... | ... | ... | " | 0.454 |
| Volume of oxygen absorbed | ... | ... | ... | ... | c.c. | 2376 |
| Respiration coefficient | ... | ... | ... | ... | ... | 1.21 |
| Mycelium (weight) | ... | ... | ... | ... | gm. | 1.279 |
| " (carbon) | ... | ... | ... | ... | per cent. | 53.6 |

TABLE XV.—Carbon balance sheets for species of *Epicoccum*.

| Species of <i>Epicoccum</i> : | | | | | | <i>Epicoccum</i> species. | <i>Epicoccum</i> species from wheat. |
|--|-----|-----|-----|-----|-----------|---------------------------|--------------------------------------|
| Catalogue number : | | | | | | Ag. 57 | Ag. 32 |
| Experiment number : | | | | | | A 2 | A 8 |
| Incubation period in days : | | | | | | 44 | 57 |
| <i>Carbon Balance Sheet.</i> | | | | | | | |
| Carbon in solution (start) | ... | ... | ... | ... | gm. | 4.834 | 5.018 |
| Carbon in H ₂ SO ₄ | ... | ... | ... | ... | " | 0.006 | 0.011 |
| " in CO ₂ ... | ... | ... | ... | ... | " | 1.333 | 1.587 |
| " in mycelium | ... | ... | ... | ... | " | 0.547 | 0.264 |
| " in solution (end) | ... | ... | ... | ... | " | 2.857 | 3.081 |
| " accounted for | ... | ... | ... | ... | " | 4.743 | 4.943 |
| " accounted for | ... | ... | ... | ... | per cent. | 98.1 | 98.5 |
| <i>Analysis of Solution.</i> | | | | | | | |
| Carbon in residual glucose | ... | ... | ... | ... | gm. | 1.642 | 2.206 |
| " in CO ₂ in solution | ... | ... | ... | ... | " | 0.017 | 0.002 |
| " in volatile acids | ... | ... | ... | ... | " | 0.037 | 0.108 |
| " in non-volatile acids | ... | ... | ... | ... | " | 0.088 | 0.149 |
| " in volatile neutral compounds | ... | ... | ... | ... | " | 0.613 | 0.297 |
| " in synthetic compounds | ... | ... | ... | ... | " | 0.063 | 0.154 |
| Total carbon accounted for | ... | ... | ... | ... | " | 2.460 | 2.916 |
| " " in solution | ... | ... | ... | ... | " | 2.857 | 3.081 |
| Carbon unaccounted for (by difference) | ... | ... | ... | ... | " | 0.397 | 0.165 |
| <i>Residual Glucose.</i> | | | | | | | |
| Glucose (by polarimeter) | ... | ... | ... | ... | per cent. | 0.825 | 1.119 |
| " (SHAFFER-HARTMANN) | ... | ... | ... | ... | " | 0.821 | 1.103 |
| " (WOOD-OST) | ... | ... | ... | ... | " | 0.832 | 1.100 |
| " (by alkaline iodine) | ... | ... | ... | ... | " | 0.870 | 1.139 |
| <i>Acids.</i> | | | | | | | |
| Titration (N/1 acid) | ... | ... | ... | ... | c.c. | 1.1 | 3.7 |
| Volatile acids (N/1 acid) | ... | ... | ... | ... | " | 1.69 | 3.74 |
| Barium salts (weight) | ... | ... | ... | ... | gm. | 0.176 | 0.417 |
| Calcium salts (weight) | ... | ... | ... | ... | " | 0.412 | 0.606 |
| Volume of oxygen absorbed | ... | ... | ... | ... | c.c. | 1537 | 2558 |
| Respiration coefficient | ... | ... | ... | ... | ... | 1.64 | 1.16 |
| Mycelium (weight) | ... | ... | ... | ... | gm. | 0.995 | 0.519 |
| " (carbon) | ... | ... | ... | ... | per cent. | 55.0 | 50.9 |

Discussion of results obtained.

Only a relatively small proportion of the carbon balance sheets presented in this paper, from a large variety of fungi, show no biochemically interesting features at all, though in most cases it is impossible to generalize because the number of species investigated in any particular genus is often small. However, of the species examined it is possible to say that none of the following gives an appreciable amount of any metabolic product from glucose other than CO_2 , and they are thus biochemically uninteresting and not promising for further intensive examination.

Class : ASCOMYCETES.

Genus 2. The only species of *Chaetomium* investigated.

Genus 3. The only species of *Sclerotinia* investigated.

Class : BASIDIOMYCETES.

Genus 1. Both species of *Ustilago* investigated.

Order : HYPHOMYCETALES.

Family : MONILIACEÆ.

Genus 2. Both species of *Sporotrichum* investigated.

Genus 4. The only species of *Cephalothecium* investigated.

Family : DEMATIACEÆ.

Genus 1. All 5 species of *Cladosporium* investigated.

The only item of interest from a biochemical point of view in the carbon balance sheets of a certain number of other species is the production of varying amounts of "carbon in volatile neutral compounds" (probably ethyl alcohol), and correspondingly high respiration coefficients. These species also are not very attractive for further investigation.

Class : ASCOMYCETES.

Genus 1. The only species of *Sordaria* investigated.

Order : HYPHOMYCETALES.

Family : MONILIACEÆ.

Genus 3. Both species of *Trichoderma* investigated.

Family : DEMATIACEÆ.

Genus 2. Four species of *Helminthosporium* out of six investigated.

Genus 4. All three species of *Alternaria* investigated.

Family : TUBERCULARIACEÆ.

Genus 2. Both species of *Epicoccum* investigated.

These two species have other items of minor interest, *e.g.*, moderately high "carbon in volatile acids" but, like the *Fusaria* (see Part V), they are not generally interesting.

The remainder of the species examined have some points of particular biochemical interest, and most of them give promise of repaying further intensive examination. These species include the following :—

Class : ASCOMYCETES. None.

Class : BASIDIOMYCETES. None.

Order : HYPHOMYCETALES.

Family : MONILIACEÆ.

Genus 1. Both species of *Eidamia* investigated.

These two species show characteristics strongly reminiscent of some of the *Aspergillus niger* group, *i.e.*, high "carbon in volatile neutral compounds," moderate titratable acidity, "carbon in non-volatile acids" and "carbon unaccounted for." They might repay further examination, but are not so promising as some of the other species dealt with in this paper.

Family : DEMATIACEÆ.

Genus 2. *Helminthosporium*. One species of six investigated gives a high figure for "carbon unaccounted for."

Genus 3. *Heterosporium*. One species of two investigated gives a moderate figure for "carbon unaccounted for."

Genus 5. *Fumago*. Both species investigated give—

(a) a high figure for "carbon unaccounted for."

(b) high titratable acidity and "carbon in non-volatile acids."

Genus 6. *Clasterosporium*. Both species investigated give good figures for "carbon unaccounted for." One species, Ag. 64, gives a very high figure.

Genus 7. *Rhacodium*. The only species investigated produces a quantity of an optically active compound, possibly a non-volatile acid.

This family includes the largest proportion of biochemically interesting genera and species of any investigated in this paper, and if a renewal of the collection of carbon balance sheets is contemplated, it seems advisable to collect a number of species of this family, and of the specified genera, for investigation.

Family : STILBACEÆ.

Genus 1. *Stysanus*. The only species investigated gives a fairly high figure for "carbon unaccounted for."

It is evident from the above facts that the biochemically interesting fungi are by no means confined to the commoner genera and species—such as species of *Aspergillus* and *Penicillium*—but that investigation of some of the rarer species, many of which grow well on purely synthetic media, is bound to lead to results of interest and to the isolation of interesting, and possibly new, types of metabolic products.

Summary.

Carbon balance sheets are given for a wide variety of species of fungi belonging to different orders, families and genera. A number of these species are evidently of biochemical interest and should be further investigated. These are dealt with in some detail in the discussion of results obtained.

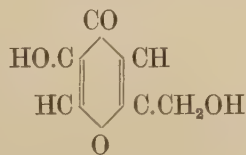
Studies in the Biochemistry of Micro-organisms.

PART VII.—*Kojic Acid (5-Hydroxy-2-Hydroxymethyl-γ-Pyrone).*

By JOHN HOWARD BIRKINSHAW, JOHN HENRY VICTOR CHARLES, CECIL HERBERT LILLY and HAROLD RAISTRICK.

IN 1907 SAITO isolated from the finely powdered mycelium of *Aspergillus oryzae*, grown on steamed rice, an organic acid which he was unable to identify, but which, from a general consideration of its chemical properties, he supposed to be β-resorcylic carbonic acid. YABUTA (1912), from the results of analysis, ascribed to the acid the empirical formula $C_{12}H_{14}O_8$, and gave to it the name "kojic acid." He described its preparation from the mycelium of *Aspergillus oryzae*, grown on rice, and also from "Koji," and pointed out that it gives an intense wine red colour with ferric chloride solution, which may be recognized even at a dilution of 1 : 200,000. Of interest from a mycological point of view is YABUTA'S observation that kojic acid is produced by a few other species of *Aspergillus*, but not by the majority, nor by species of *Penicillium* or *Mucor*.

The same author later (1916, 1922) stated that the acid is formed in 10 per cent. yield, when *Aspergillus oryzae* is grown in a 10 per cent. solution of dextrose with salts, but experimental details are lacking. As a result of further chemical investigation, and of molecular weight determinations, YABUTA altered his previous empirical formula for the new acid to $C_6H_6O_4$. By the preparation of various derivatives he also came to the unproven but very probable conclusion that its constitution is 5-hydroxy-2-hydroxymethyl-γ-pyrone. Later (1924), working in BARGER'S laboratory, he definitely proved this constitution to be correct, and established the formula for kojic acid as :—



As noted by BARGER, in a footnote to YABUTA'S 1924 paper, the present writers independently discovered kojic acid in 1923, as a direct outcome of the quantitative experiments described in Part III of this series, among the metabolic products of various species of *Aspergillus*, and in particular of *Aspergillus parasiticus* SPEARE. An account of this work is given in the following pages.

Other workers have also isolated what is very probably kojic acid from other *Aspergilli*. Thus TRAETTA-MOSCA (1914) isolated a substance having the formula $C_6H_6O_4$, formed from sucrose, glucose or lævulose by a fungus to which he gave the name *Aspergillus glaucus*, which, from its properties, melting point, and the melting points of its derivatives, is undoubtedly kojic acid. TRAETTA-MOSCA considered the new compound to be the γ -lactone of a trihydroxy-hexadienoic acid. Later TRAETTA-MOSCA and PRETI (1921) obtained the same substance by the action of the same mould on glycerol. WIJKMANN (1924) also obtained a similar substance as a result of the growth of *Aspergillus* sp. on media containing sucrose.

Finally, the colour reaction—production of a wine red colour with ferric chloride—reported by THOM and CHURCH (1921) as being given by cultures, on a liquid starch medium, of *Aspergillus tamarii*, *flavus* and *oryzæ* is doubtless also due to kojic acid.

In the quantitative work on the metabolic products of different species of *Aspergillus* grown on a liquid glucose medium (see Part III), it was found, when the carbon balance sheet was prepared for *Aspergillus parasiticus* SPEARE, that over 19 per cent. of the sugar consumed was unaccounted for in the end products estimated (see Table XIII, p. 48). It was also noticed that when the metabolism solution which was being analysed for “synthetic carbon” had been treated with colloidal ferric hydroxide and then heated, the filtrate was a deep orange red in colour. When the original solution was tested with ferric chloride a deep wine red colour appeared. The substance giving this colour was found to be extracted slowly by ether. The residues from the metabolism experiment were, therefore, extracted with ether, and yielded a small amount of crystals, which, after recrystallisation and sublimation in a high vacuum, melted at 151°C . In view of the resemblance between our product and YABUTA's kojic acid, a further supply was prepared, and its reactions and derivatives compared with those of kojic acid as described by YABUTA. This established beyond doubt the identity of our substance with kojic acid as is shown by the following figures:—

| | | | | | Observed. | YABUTA |
|-----------------------------|----|----|----|----|-----------------------|-----------------------|
| Original substance, m.pt. | .. | .. | .. | .. | 152°C . | 152°C . |
| Diacetyl derivative, m.pt. | .. | .. | .. | .. | 103°C . | 102°C . |
| Dibenzoyl derivative, m.pt. | .. | .. | .. | .. | 135°C . | 136°C . |

Ultimate analysis:—

| | | | | | | Observed. | Calculated. |
|----------|----|----|----|----|----|-----------|-------------|
| | | | | | | Per cent. | Per cent. |
| Carbon | .. | .. | .. | .. | .. | 50.56 | 50.70 |
| Hydrogen | .. | .. | .. | .. | .. | 4.61 | 4.26 |

The Production of Kojic Acid by different Species of Aspergillus.

The ferric chloride colour reaction given by kojic acid is so delicate, and so easy to perform, that it was decided to carry out a series of tests on all the species of *Aspergillus*

available, primarily with a view to obtaining the most suitable species of *Aspergillus* for the preparation of kojic acid, and, secondly, to investigating the possible diagnostic value of the test for differentiating species or groups of *Aspergillus*.

For this purpose a quantity of the usual CZAPEK-DOX medium having the composition given on p. 7 was made up.

This was tubed out in 10 c.c. quantities, allowing six tubes for each species of fungus investigated. All the cultures of *Aspergillus* in stock, numbering 95, a list of which is given in Part III, were sown from single spore cultures into this medium, incubated at 24° C. in the dark, and the ferric chloride test was carried out on a separate tube culture of each species at intervals of 3, 6, 12, 18, 24 and 48 days. The test was performed by adding to the culture three drops of 10 per cent. ferric chloride solution. In the presence of even traces of kojic acid a red colour is obtained, while with the typical kojic acid formers a most intense wine red colour is produced. It is noteworthy that various not very definite colour changes were observed with other fungi, but these cannot be confused with the typical colour given by kojic acid.

Of the 95 species of *Aspergillus* examined, a positive test for kojic acid was given only by the following species :—

1. *Aspergillus parasiticus* SPEARE. National collection of Type Cultures, No. 975. Ac. 14.
2. *Aspergillus oryzae* AHLBERG. National Collection of Type Cultures, No. 598. Ac. 19.
3. *Aspergillus effusus* TIRABOSCHI. National Collection of Type Cultures, No. 973. Ac. 21.
4. *Aspergillus tamarii* KITA. National Collection of Type Cultures, No. 599. Ac. 26.
5. *Aspergillus* sp. D.20, obtained from Mr. F. T. BROOKS, Cambridge, and identified as a strain of *Aspergillus tamarii*, Ac. 62.
6. *Aspergillus* sp., isolated at Ardeer from mouldy "Pegamoid" and identified as a strain of *Aspergillus flavus*. Ac. 91.

In no case was a positive reaction given in three days, while in twelve days each of the above six cultures except *Aspergillus oryzae* gave a positive test, and in eighteen days each gave an intense colour. This persisted for a long time, but after a lapse of 48 days was beginning to diminish.

So far as the first object of these experiments is concerned, it was decided that *Aspergillus parasiticus* SPEARE was the most suitable of all our cultures for the preparation of kojic acid, since it seemed to give the most intense colour reaction, and also grew very well on the culture medium used.

It appears certain that the kojic acid test has a definite diagnostic value for the

differentiation of the *Aspergilli*, provided the test is carried out as described above, and, in particular, provided the same culture medium is used. As will be seen, the only species of *Aspergillus* giving kojic acid under the above conditions are *Aspergillus flavus*, *oryzæ*, *parasiticus*, *effusus*, and *tamarii*. Of these the first four are all included morphologically in the *A. flavus-oryzæ* group. Thus THOM and CHURCH (1921, p. 113) say, "*A. oryzæ*, *A. parasiticus*, and *A. effusus* are morphologically recognizable varieties or species which are certainly closely related to the cosmopolitan group of which the organism described by BREFELD and WEHMER and believed by them to be *A. flavus* LINK, may be called the type." *A. tamarii* is also closely related to the *A. flavus-oryzæ* group, and in this connection it is interesting to note that not only are our biochemical tests entirely confirmatory of the morphological opinion of THOM and CHURCH expressed above, but also of similar biochemical tests carried out by them. Thus they say (*loc. cit.*, p. 121), "Tests for phenolic substances with an aqueous solution of ferric chloride align the *A. tamarii* group closely with the *A. flavus-oryzæ* group as to this particular chemical reaction. All strains of these three species which were tested showed a red reaction, varying from brownish red to a rich wine red."

It may, therefore, be accepted that the kojic acid test is of diagnostic value in that it justifies one in placing in the *flavus-oryzæ-tamarii* group of *Aspergilli* any species of this genus which, if cultured under the conditions above specified, gives rise to a typical kojic acid reaction, without necessarily excluding from this group any *Aspergillus* which gives a negative reaction.

This reservation is made since of two cultures of *A. flavus* in stock, the one strain—Ac. 91—gives a positive kojic acid reaction, and the other, Ac. 16, a negative reaction. The latter culture, *A. flavus* BREFELD was obtained from the National Collection of Type Cultures (Catalogue No. 596), which obtained it originally from the Centralstelle, Amsterdam.

The test is further restricted in value, however, since we have recently shown that kojic acid is produced from glucose by a single species of *Penicillium* in our collection, though not by any other of the 120 species of *Penicillium* in this collection. This species—Catalogue No. Ad. 116—was purchased in 1929 from Baarn, labelled *P. lanosum* WESTLING. A single spore culture was made and used, and a sub-culture sent to Dr. CHARLES THOM, who reported, "I am confident that this species is *P. Daleæ* ZALESKI, in quite a different section from *P. lanosum*."

On cultivating this strain of *P. Daleæ* ZALESKI, on the usual CZAPEK-DOX medium in the dark at 24° C., a negative kojic acid reaction with ferric chloride solution was given after 10 days' or 20 days' incubation, but after 30 days a typical colour reaction was obtained. All doubts as to the nature of the product giving rise to this colour reaction were removed by the isolation from flask cultures on CZAPEK-DOX solution of a considerable quantity of crystals having the same m.pt. and mixed m.pt. as kojic acid, and giving 50.63 per cent. carbon and 4.31 per cent. hydrogen. (Theoretical for $C_6H_6O_4$, carbon = 50.70 per cent., hydrogen 4.26 per cent.) The crystals further

gave a diacetyl compound melting at 101°–102° C., and having a mixed melting point with diacetylkojic acid of 101°–103° C.

The results obtained by YABUTA (1912) are difficult to explain. YABUTA states, without giving details of the medium on which the fungi are grown, that kojic acid is produced by *Aspergillus oryzae*, *albus*, *candidus*, and *nidulans*, but not by *Aspergillus glaucus*, *Okazakii*, *melleus*, *minimus*, *fumigatus*, *Wentii*, *niger*, *ochraceus*, *luchuensis*, *flavus*, *clavatus*, *giganteus* and *varians*. The species giving negative results are in agreement with our own findings, but there are marked discrepancies between our positive results. Thus, while YABUTA obtains positive results with *Aspergillus oryzae*, *albus*, *candidus*, and *nidulans*, a colour reaction was only obtained in the present investigation with the first of these four species. There is only one named culture of *A. albus* and of *A. candidus* at Ardeer, each of which was obtained from Baarn, but there are in addition eleven other white species, nine of which were obtained from THOM and CHURCH, and two were isolated in this laboratory, yet the whole thirteen white species gave entirely negative results. Similarly no positive kojic acid test was obtained with five authentic cultures of *Aspergillus nidulans*—one obtained from Baarn, one from PRIBRAM, two from Miss CHURCH, and one isolated in this laboratory and identified by Miss CHURCH. If, therefore, the authenticity of YABUTA's cultures is accepted, the most probable explanation of the discrepancies in our findings seems to be that YABUTA cultivated his fungi on a different medium and under different conditions from ours. For this reason it cannot, in our opinion, be too strongly stated that, if the test is to have any diagnostic value, it must be carried out exactly as described.

Fortunately there seems to be enough internal evidence in TRAETTA-MOSCA's paper (1914) to explain his statement that his compound $C_6H_6O_4$, which is almost certainly kojic acid, is produced by *Aspergillus glaucus*. This is contradictory, not only to our findings, but to those of YABUTA, and is to be explained by the fact that, in our opinion, TRAETTA-MOSCA's culture was not *Aspergillus glaucus*, but a member of the *A. flavus-oryzae* group. This opinion is based on the following facts:—

- (1) TRAETTA-MOSCA states that his culture gives rise to ethyl alcohol in addition to kojic acid. The quantitative experiments described in Part III prove definitely that no alcohol is produced by any member of the *A. glaucus* group, but is given in quantity by all the members of the *A. flavus-oryzae* group tested.
- (2) TRAETTA-MOSCA cultivated his organism at 37° C. None of our cultures of the *A. glaucus* group will grow at all at 37° C., while all our cultures of the *A. flavus-oryzae* group grow well at this temperature.
- (3) None of our cultures of the *A. glaucus* group gives a positive reaction for kojic acid with ferric chloride.

The Production of Kojic Acid from Different Sources of Carbon.

The mode of formation of kojic acid from carbohydrates by fungi is of considerable biochemical interest because of the fact that the γ -pyrone nucleus is a common constituent of so many groups of naturally occurring chemical compounds. With a view to obtaining some information on this point, a series of tests, detailed below, was carried out on the production of kojic acid from different sources of carbon.

A CZAPEK-DOX medium (Medium A) was made up of the same salt composition as that described on p. 7. To separate lots of this medium 5 per cent. of one of the following compounds was then added in place of the usual 5 per cent. glucose: l  vulose, sucrose, galactose, lactose, xylose, arabinose, glycerol, mannitol, starch. The different batches were tubed out in quantities of 10 c.c., sterilized, and 6 tubes of each batch sown with each of the six species mentioned on p. 129 as giving a positive test for kojic acid when grown on a medium containing glucose. The strain of *Aspergillus flavus*, catalogue number Ac. 16, which gave a negative test for kojic acid on glucose, was also included. The cultures were incubated at 24   C. in the dark, and at intervals of 3, 6, 12, 18, 24 and 48 days, one tube for each species of *Aspergillus* from each batch of medium was tested for kojic acid by adding to it three drops of 10 per cent. ferric chloride solution.

A similar experiment was also carried out in which the composition of the medium was very slightly varied. In this medium (Medium B in Table I) 0.2 per cent ammonium nitrate was used in place of 0.2 per cent. sodium nitrate present in Medium A (see p. 7). In all other respects the two experiments were exactly similar.

The best results were usually obtained after 24 days' incubation, and are recorded for both experiments in Table I. In the cases of lactose and mannitol, however, the results after 48 days' incubation are incorporated in place of those obtained at 24 days, since only scanty growths and doubtful kojic acid tests were obtained on these two compounds after 24 days' incubation. Better growths and more decisive tests were obtained after 48 days.

It will be seen from Table I that the strain of *A. flavus* (Ac. 16) gave entirely negative results on all the ten compounds tried, both in Medium A and in Medium B, nor did this strain give a positive reaction at any other period of its growth, as the other tests at different time intervals showed. On the other hand, another strain of *A. flavus* (Ac. 91) gave a positive kojic acid test on all the sources of carbon except lactose.

The results obtained with Medium A and Medium B show definite differences, and indicate anew the need for media having a definite, easily reproducible chemical composition in comparative work of this description.

It is evident that kojic acid is produced by one or other of the fungi investigated from all the ten sources of carbon supplied. These include a polysaccharide (starch), two disaccharides (sucrose and lactose), three hexoses (glucose, l  vulose, galactose), two pentoses (xylose, arabinose), one hexahydric alcohol (mannitol) and one

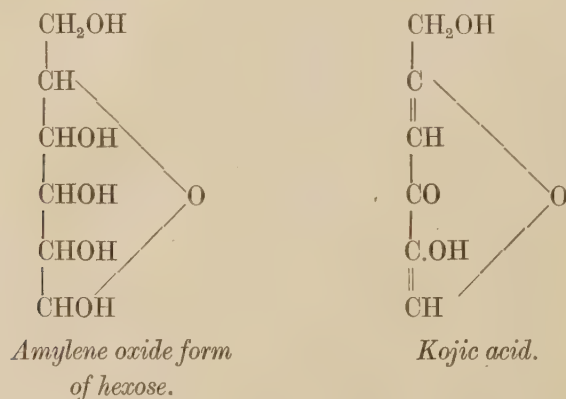
TABLE I.—Results of Kojic Acid Test after 24 days' Incubation at 24° C.
Medium A contained NaNO₃. Medium B contained NH₄NO₃.

| Culture. | Medium. | Glucose. | Levulose. | Sucrose. | Galactose. | Lactose (after 48 days). | Xylose. | Arabinose. | Glycerol. | Mannitol (after 48 days). | Starch. |
|-------------------------------|---------|----------|-----------|----------|------------|--------------------------------|---------|------------|-----------|---------------------------------|---------|
| <i>A. parasiticus</i> Ac. 14 | A | +++ | ++ | +++ | + | — | + | ++ | ++ | +++ | + |
| | B | +++ | +++ | +++ | +++ | — | +++ | + | +++ | +++ | + |
| <i>A. flavus</i> Ac. 16 | A | — | — | — | — | — | — | — | — | — | — |
| | B | — | — | — | — | — | — | — | — | — | — |
| <i>A. oryzae</i> , Ac. 19 | A | + | — | — | + | — | — | — | — | — | — |
| | B | ++ | — | — | — | — | — | — | — | — | — |
| <i>A. effusus</i> , Ac. 21 | A | +++ | + | +++ | +++ | + | ++ | + | ++ | + | + |
| | B | +++ | — | +++ | +++ | + | +++ | — | — | — | — |
| <i>A. tamaris</i> , Ac. 26 | A | +++ | ++ | +++ | + | — | ++ | + | ++ | ++ | + |
| | B | +++ | +++ | +++ | +++ | — | +++ | ++ | +++ | +++ | + |
| <i>A. tamaris</i> , Ac. 62... | A | +++ | + | +++ | + | ++ | + | + | + | + | ++ |
| | B | +++ | +++ | +++ | +++ | + | +++ | + | +++ | + | + |
| <i>A. flavus</i> , Ac. 91 | A | +++ | + | +++ | ++ | — | ++ | + | ++ | ++ | + |
| | B | +++ | +++ | +++ | +++ | — | +++ | + | +++ | +++ | — |

+++ = intense reaction; ++ = strong reaction; + = positive reaction.

trihydric alcohol (glycerol). Hence the idea originally held, that kojic acid ($C_6H_6O_4$) arises from glucose ($C_6H_{12}O_6$) by a simple process of abstraction of water and oxidation, offers no explanation of the formation of kojic acid from compounds containing less than six carbon atoms in the molecule, *e.g.*, xylose, arabinose, and glycerol. Either of the following explanations seems to offer a possible solution of the difficulty, but there is at present no conclusive experimental evidence as to whether either is correct :—

- (1) In common with some other types of fermentation, as shown for the latter by NEUBERG and his co-workers, acetaldehyde may be produced by the fungus from the carbon source supplied whether this be a poly-, di-, or monosaccharide, a pentose, or a polyhydric alcohol. The acetaldehyde may then be condensed by a series of reactions to kojic acid. This explanation is apparently supported by the fact that it has been shown that all those fungi which produce kojic acid also produce at the same time ethyl alcohol, and hence, in passing, acetaldehyde.
- (2) The source of carbon supplied, whatever its nature, may be first anabolised by the fungus into a reserve carbohydrate, which is later hydrolysed by the micro-organism, as occasion arises, into a monosaccharose which in its turn gives rise to kojic acid. The close similarity between the amylenoxide form of a 6-carbon sugar, and kojic acid



renders this a probable explanation, and it is further supported by the fact that various fungi are known to store reserve carbohydrates and utilise them later as occasion demands, *e.g.*, glycogen in yeast, trehalose in *A. niger*.

A definite proof was obtained that kojic acid itself is produced from xylose, and is really responsible for the colour reaction with this carbohydrate, by growing *A. effusus* on 500 c.c. CZAPEK-DOX solution containing 5 per cent. of xylose. This medium was distributed in two 750 c.c. conical flasks, and incubated for eleven days at 23° C. The liquid was filtered from the mycelium, evaporated to small bulk and extracted with ether in a continuous extraction apparatus. The crystals separating from the ether were recrystallised from acetone, and had then a melting point of 152° C. Total yield,

1.76 gm. The product had still the same melting point after mixing with it some kojic acid prepared from glucose, and is evidently identical with kojic acid.

Preparation of Kojic Acid.

Since kojic acid is readily prepared, and, being a pyrone derivative, is of considerable chemical interest, it may be of value to describe in detail the method found most suitable for its production in some quantity (500 gm.). In order to do this conveniently it was found necessary to design apparatus suitable for the cultivation of a fungus, in pure culture, on quantities of culture medium amounting to 60 litres, and in shallow layers so as to offer a large surface for growth. Since no stock apparatus was available for this purpose, a combined incubator-steriliser was designed and built in this laboratory, and, as this may be of interest to others wishing to cultivate fungi on a scale larger than that generally used in the laboratory, a description of it is appended (p. 136).

Smaller amounts of kojic acid, 30–40 gm., may conveniently be prepared by cultivating separate 350 c.c. quantities of medium in about a dozen 1-litre conical flasks, and following the method described later for the isolation of the kojic acid produced.

60 litres of medium were made of the following composition :—

| | | | | | | | | gm. |
|---------------------------------------|----|----|----|----|----|----|----|------------|
| NaNO ₃ | .. | .. | .. | .. | .. | .. | .. | 2.0 |
| KH ₂ PO ₄ | .. | .. | .. | .. | .. | .. | .. | 1.0 |
| KCl | .. | .. | .. | .. | .. | .. | .. | 0.5 |
| MgSO ₄ . 7H ₂ O | .. | .. | .. | .. | .. | .. | .. | 0.5 |
| FeSO ₄ . 7H ₂ O | .. | .. | .. | .. | .. | .. | .. | 0.01 |
| Sucrose | .. | .. | .. | .. | .. | .. | .. | 50.0 |
| Water | .. | .. | .. | .. | .. | .. | .. | 1,000 c.c. |

Sucrose was used because it gives as good or slightly better yields of kojic acid than glucose, and is more easily and cheaply obtained in quantity in the pure state. Five litres of this medium were placed in each of the twelve incubating trays of the apparatus described on p. 136, giving a depth of liquid of about 1 inch, the incubator closed, and sterilized by steaming. After cooling, each tray was sown with the spores from a beer-wort agar ROUX bottle culture of *Aspergillus parasiticus* SPEARE (Ac. 14). The spores were emulsified in sterile distilled water and introduced by means of a sterile pipette through the apertures provided for inoculation at the side of the cabinet. By means of a sterile bent glass rod, the spores were then floated over the whole surface of the medium. The mould was cultivated at room temperature for eight weeks. This relatively long incubation period was necessary as the average room temperature was very low, the experiment being carried out during the winter months. At 24° C. the incubation period is only about four weeks. During the whole incubation period a continuous stream of sterile air was passed through the apparatus and it should be

remarked here that to ensure a good yield of kojic acid it is essential that the culture should be thoroughly aerated. From time to time samples of the liquid were withdrawn through the inoculating apertures, the sugar content estimated and the kojic acid determined by the method described in Part VIII of this series. At intervals the cultures were examined through the inspection windows for contaminations, but at no time was any trouble from infection experienced. When the yield of kojic acid had reached a maximum, as much as possible of the liquid was syphoned off through the inoculating apertures, from below the mycelium, and filtered, and then the whole of the contents of the incubator were sterilized by steaming. The liquid remaining in the trays was filtered from the dead mycelium and the combined filtrates evaporated at 50–60° C. in open dishes till the volume was reduced to about 10 litres. It was then filtered again, further concentrated *in vacuo* to about 2 litres, set aside to crystallise, and the crystals, yellowish brown in colour, separated by filtration through muslin. The colouring matter and other impurities were most conveniently removed by dissolving the crude kojic acid in spirit and precipitating the impurities by the addition of an equal volume of ether. After filtering off the precipitate, the ether and part of the spirit were removed from the clear filtrate by distillation and the kojic acid crystallised from the hot spirit as a pale yellow solid. The mother-liquors and residues from recrystallisation were treated with ether in a HURTLEY continuous extraction apparatus until they gave only a slight reaction with ferric chloride, and the extracted kojic acid recrystallised from spirit.

The total amount of recrystallised product was 563 gm. while the calculated yield from a kojic acid estimation on one dish was 700 gm. In another experiment, using glucose, 505 gm. of kojic acid were obtained from an estimated yield of 620 gm. These figures represent recovery yields, calculated on sugar, of 18·8 per cent. and 16·8 per cent. from sucrose and glucose respectively.

Kojic acid when quite pure is perfectly white, but during isolation it sticks tenaciously to small amounts of colouring matter, so that the final product as obtained above is slightly coloured. From this a perfectly pure white product is most readily prepared by sublimation *in vacuo*, followed by recrystallisation from ether.

The presence of citric acid, formed during the fermentation, may be readily demonstrated in the residues from the continuous ether extraction. The residues, which are strongly acid, are neutralised with sodium hydroxide, an excess of calcium chloride solution added, the mixture heated to boiling for some time, and filtered hot. Under these conditions calcium citrate is precipitated and from this citric acid may readily be obtained.

Description of Large-scale Laboratory Apparatus for the Cultivation of Moulds.

The apparatus, dimensions of which are given in the illustrations on pp. 366–367, consists of a rectangular box or cabinet of mild steel plates, welded together, one side being removable, in two halves, to serve as doors. The doors are held in position by

means of studs and wing nuts, rubber insertion being placed between the doors and round the edge of the cabinet to ensure a close joint.

The box or cabinet is provided with 12 shelves (arranged in two tiers of six) of perforated aluminium, each capable of supporting a large photographic dish (approximate dimensions being 21 inches \times 17 inches \times 2½ inches). Bolted to the underside of each shelf, and extending over a slightly greater area than that occupied by the dish below, is a cover of thin sheet aluminium, dished so as to allow condensed steam to collect and finally fall beyond the outer edges of the dish below, thus obviating dilution of the medium contained therein.

At each end of the cabinet, and corresponding with each shelf, an aperture is provided, with suitable metal collar, to hold a plug, cork or thermometer, and through which a pipette or glass tube can be inserted at an angle of about 45° for the purpose of either inoculating the medium or withdrawing it from the dishes when required. In addition, a narrow plate glass inspection window is provided at each end to facilitate observation.

At the bottom of the cabinet, at one end, a drain cock is provided for drawing off condensed steam. At the other end a perforated steam pipe enters and traverses the length of the cabinet, below the bottom shelves. At the point where this steam pipe enters the cabinet a T-piece is provided so that connection can be made to both steam and air supplies, thus obviating risk of infection and necessity for disconnecting when either steam or air are required, the same pipe being used for steam sterilizing the cabinet and its contents, and subsequently for the supply of washed and filtered air.

The whole apparatus is supported on a SLINGSBY bogey so that, if necessary, it can be transferred from place to place.

The following is a brief description of the procedure adopted when putting the apparatus into commission.

Twelve dishes, each containing about 5 litres of medium, are placed inside the cabinet; the doors are bolted on, cotton wool plugs and one or two thermometers at different points are then inserted into the openings provided for subsequent inoculation purposes. Steam, after passing through a suitable steam trap to remove water, is fed into the cabinet until a temperature of 100° C. has been reached and maintained for two to three hours. This operation is performed on three successive days, the medium being inoculated on the fourth day.

During the fermentation process air is supplied to the cabinet after passing through wash bottles containing :—

1. Water.
2. Potassium permanganate.
3. Sulphuric acid.
4. Sodium hydroxide.

At the end of the fermentation process the medium is syphoned from the dishes and steam is again passed into the cabinet for an hour, after which the doors may be removed

and the mould films dealt with without unduly distributing infection throughout the laboratory.

Summary.

Kojic acid (5-hydroxy-2-hydroxymethyl- γ -pyrone)— $C_6H_6O_4$ —was, as a result of the quantitative metabolism experiments previously described, isolated from the metabolic products of a small number of *Aspergilli*. The production of kojic acid is shown to be characteristic of species of the *A. flavus-oryzæ* group and serves as a useful diagnostic test for species of *Aspergillus* belonging to this group.

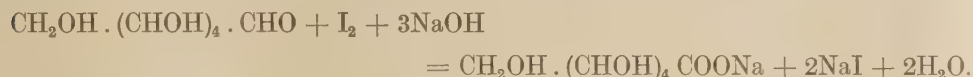
Kojic acid is produced by these fungi from a large number of carbon compounds—*e.g.*, mono- and polysaccharides, and also from some compounds containing three and five carbon atoms in the molecule, *e.g.*, glycerol and xylose. The significance of this, in considering the mechanism of the formation of kojic acid, is discussed.

Details are given for the preparation of kojic acid in quantity, and a description is appended of an apparatus specially designed for the purpose of growing fungi in pure culture on a relatively large scale.

*Studies in the Biochemistry of Micro-organisms.*PART VIII.—*The Estimation of Kojic Acid.*

By JOHN HOWARD BIRKINSHAW and HAROLD RAISTRICK.

In the estimation of the residual glucose in the metabolic products of *Aspergillus parasiticus* SPEARE, it was found that very much lower results were given by the copper reduction method of SHAFFER and HARTMANN (1921) than by the iodine absorption method of HINTON and MACARA (1924). In the latter method, which in all essential details is the same as that previously described by WILLSTÄTTER and SCHUDEL (1918), glucose is oxidized by iodine in alkaline solution, the only product arising from the glucose being gluconic acid (as the sodium salt) according to the equation:



It was noticed during the estimation of the glucose in the *A. parasiticus* experiment that an immediate turbidity and a strong smell of iodoform were produced after the addition of the iodine and sodium hydroxide to the metabolism solution, thus indicating that some other reaction was occurring besides the oxidation of glucose to gluconic acid. Since kojic acid was known to be present among the metabolic products of *A. parasiticus* on glucose it was considered that this might be the cause of the anomalous results. A test with pure kojic acid showed that this was the case.

As no method save a rough colorimetric one was available for the estimation of kojic acid, the reaction was further investigated with a view to its utilisation for this purpose, and it was found that, when the conditions of HINTON and MACARA's method were suitably modified, a quantitative relationship existed between the iodine used and the kojic acid present.

This was established as follows:—An aqueous solution of pure kojic acid was prepared containing 1.4538 gm. kojic acid per litre. Measured volumes of this solution were then treated with alkaline iodine solution (40 c.c. of N/10 iodine and 50 c.c. of N/10 sodium hydroxide) for different lengths of time, and the results of a series of these estimations are given in Table I.

The figures in column 4 give the weight in grams of kojic acid equivalent to 1000 c.c. of N/1 iodine, under the conditions of the test and are calculated from columns 2 and 3.

TABLE I.—Absorption of Iodine by different amounts of Kojic Acid Solution (1.4538 gm. per litre) after varying time intervals.

| Time in minutes. | Cubic centimetres of kojic acid solution used. | Cubic centimetres N/10 iodine absorbed. | Gm. of kojic acid equivalent to 1000 c.c. N/1 iodine. | Atoms of iodine absorbed by 1 molecule of kojic acid. |
|------------------|--|---|---|---|
| 120 | 25.02 | 23.80 | 15.28 | 9.29 |
| 60 | 20.10 | 19.67 | 14.86 | 9.56 |
| 90 | 20.10 | 19.82 | 14.74 | 9.63 |
| 120 | 20.10 | 19.74 | 14.80 | 9.60 |
| 60 | 10.02 | 10.09 | 14.44 | 9.83 |
| 90 | 10.02 | 10.21 | 14.27 | 9.95 |
| 120 | 10.02 | 10.21 | 14.27 | 9.95 |
| 120 | 5.00 | 5.12 | 14.20 | 10.00 |

The figures in column 5 give the number of atoms of iodine absorbed by one molecule of kojic acid (molecular weight 142), under the conditions of the test, and are calculated from column 4.

It will be seen that, provided a sufficient excess of iodine is present, and a sufficient time allowed for the reaction, kojic acid reacts quantitatively with iodine in alkaline solution, one molecule of kojic acid absorbing 10 atoms of iodine. The conditions necessary to secure this are:—

- (1) To use at least four times the quantity of iodine theoretically required.
- (2) To allow at least 90 minutes for the reaction.

These conditions were adopted as standard.

The following are the details of the method proposed for the estimation of kojic acid:—

An amount of solution containing 7 to 14 mgm. of kojic acid is pipetted into a 350 c.c. conical flask and the volume made up to 100 c.c. with distilled water from a measuring cylinder. 40 c.c. of N/10 iodine are added from a pipette followed by 50 c.c. of N/10 sodium hydroxide from a cylinder. The mixture is shaken and set aside for 90 minutes, the flask being stoppered. At the end of this time 10 c.c. of 2 N sulphuric acid are added and the excess of iodine is titrated with N/10 sodium thiosulphate solution. The difference between this figure and a blank on the 40 c.c. of iodine alone gives the amount of iodine absorbed, and from this the amount of kojic acid in the solution can be readily calculated since 1 c.c. N/10 iodine \equiv 0.00142 gm. of kojic acid.

The method is equally applicable to the estimation of kojic acid in the presence of glucose, since it has been shown by experiment that glucose, in pure solution, may be estimated accurately by following the details given in the above method. Hence, if it is desired to estimate kojic acid in the presence of glucose, an estimation is carried out on the solution containing the two substances, exactly as described in the method detailed above. The amount of iodine absorbed gives the iodine equivalent of the sum

of the glucose and kojic acid present. The glucose is then estimated separately by any method which gives accurate results for glucose in the presence of kojic acid, and from this figure the iodine equivalent of the glucose may be calculated since 1 c.c. N/10 iodine \equiv 0.0090 gm. glucose. If this figure is deducted from the iodine equivalent previously found for the sum of the glucose and kojic acid, the iodine equivalent, and hence the amount, of kojic acid may be calculated.

If, as occasionally happens, there are present in solution along with kojic acid, alcohol, acetaldehyde, or other volatile compounds giving rise to iodoform when treated with alkaline iodine solution, it is essential to remove these from solution by distillation, before carrying out an estimation. The method is, of course, inaccurate in the presence of any non-volatile compounds which absorb iodine from alkaline iodine solution.

The method has been used with considerable success to follow the course of the production of kojic acid by certain moulds.

To complete the investigation and confirm the results so far obtained it was decided to investigate the reaction between iodine and kojic acid in more detail, in order, if possible, to obtain an equation for the reaction, and also to determine by what mechanism it arises. The work carried out with these purposes in view will be described under the headings :

(A) Equation of the Reaction between Iodine and Kojic Acid (p. 141).

(B) Mechanism of the Reaction between Iodine and Kojic Acid (p. 148).

A. Equation of the Reaction between Iodine and Kojic Acid.

Qualitative examination of the products of the reaction between iodine and kojic acid in alkaline solution showed that the following substances are formed :

- (1) iodoform
- (2) iodide
- (3) formic acid
- (4) oxalic acid
- (5) glycollic acid.

The details of this qualitative examination follow, and it will be seen that the experimental method adopted was slightly different from that employed in the quantitative method. The following changes were made :—In order to keep the volume of liquid within reasonable limits and to avoid the addition of extraneous potassium iodide, a methyl-alcoholic solution of iodine was used in place of the usual aqueous solution of iodine in potassium iodide, and, in order to avoid the introduction of elements which could not be removed later, *e.g.*, sodium or potassium, barium hydroxide was used to dissolve the iodine.

1.42 gm. of pure kojic acid were dissolved in 2,700 c.c. of water, and to this was added a solution of 25.4 gm. of iodine in 150 c.c. of pure methyl alcohol. Some of the iodine was precipitated in a finely divided state, but was immediately re-dissolved on the addition of 40 gm. of barium hydroxide crystals ($\text{Ba}(\text{OH})_2 \cdot 8 \text{H}_2\text{O}$) dissolved in 300 c.c. of water. A yellow precipitate was formed, and after standing for three hours this was filtered off and recrystallised from acetone. It had the characteristic crystalline form (hexagonal plates), smell, and melting point (119°C.) of iodoform.

250 c.c. of N/1 sulphuric acid (approximately equivalent to the baryta) were added to the filtrate from the iodoform, the precipitated barium sulphate and iodine removed by filtration, and the iodine remaining in solution extracted by shaking out several times with ether. A test applied to a little of the extracted solution, *i.e.*, acidification with nitric acid and addition of silver nitrate, showed the presence in solution of iodides, and these, together with the small amount of free iodine still remaining in solution, were removed by treatment with an excess of freshly prepared and well-washed silver carbonate. The precipitated silver iodide was filtered off, and the excess of silver removed from the filtrate by treatment with sulphuretted hydrogen.

The filtrate from the silver sulphide was evaporated *in vacuo* for a short time to remove all sulphuretted hydrogen, neutralised with baryta to obtain as barium salts any organic acids formed from the kojic acid, and the solution evaporated *in vacuo* to small bulk, after the previous removal of a slight precipitate of barium sulphate. During the evaporation a barium salt separated, and was filtered off when the volume of the liquid had been reduced to about 300 c.c. This compound was dissolved in dilute hydrochloric acid, the barium precipitated with sulphuric acid, filtered, the filtrate made slightly alkaline with ammonia, and a little calcium acetate solution added. An immediate precipitate was formed which was insoluble in acetic acid but soluble in hydrochloric acid, indicating the presence in solution of oxalic acid, which was precipitated during evaporation as barium oxalate.

The filtrate from the barium oxalate, containing in solution any soluble barium salts arising from the kojic acid, was quantitatively precipitated with sulphuric acid to remove barium, and the solution of free acids evaporated *in vacuo* almost to dryness. The distillate was strongly acid, and, on treatment with lead carbonate and filtration of the hot solution, deposited characteristic crystals of lead formate, which on analysis gave :

| | Found. | Calc. for $(\text{HCOO})_2 \text{Pb.}$ |
|----------------|-----------|--|
| | Per cent. | Per cent. |
| Lead | 69.68 | 69.71 |

Formic acid is thus one of the products of the action of iodine on kojic acid.

The solution left in the distillation flask after the removal of the formic acid set to a mass of crystals on leaving in a desiccator over concentrated sulphuric acid. As no satisfactory solvent could be found for the recrystallisation of the acid, the latter

was dissolved in water, neutralised with calcium carbonate, and the calcium salt crystallised from hot water. It had the characteristic appearance of calcium glycollate, a specimen of which was prepared and crystallised in the same way for comparison. Analysis after drying to constant weight at 110° C. confirmed the salt as calcium glycollate, and hence proved the formation of glycollic acid from kojic acid.

Calcium.

| | | |
|---------------------|-------------|-------------------|
| (By sulphated ash.) | Found | = 20.76 per cent. |
| | Theoretical | = 21.06 „ |

Carbon.

| | | |
|----------------------|-------------|-------------------|
| (By wet combustion.) | Found | = 24.68 per cent. |
| | Theoretical | = 25.26 „ |

Methods were therefore devised for the estimation of each of these reaction products—iodoform, iodide, formic, oxalic, and glycollic acids—and are described in detail below.

(1) and (2) *Estimation of Iodoform and Iodides.*—10.02 c.c. of a M/100 solution of pure kojic acid were submitted to the action of alkaline iodine as in the quantitative estimation, but, in order to avoid introducing extraneous iodide with the standard iodine solution, a special solution of hypoiodite was made up equivalent to 4N/50 in respect of iodine and N/10 in respect of sodium hydroxide. 50 c.c. of this solution were therefore equivalent to the 40 c.c. of N/10 iodine and 50 c.c. of N/10 sodium hydroxide recommended for use in the actual estimation of kojic acid. At the same time, a blank experiment was carried out on exactly the same lines, using 10 c.c. of water in place of the 10 c.c. of kojic acid solution. Curiously enough, when the hypoiodite solution and the kojic acid solution with the addition of the appropriate quantity of water were mixed, no reaction took place and the titration after 90 minutes was the same as the blank. The reaction could be induced, however, by adding to the reaction mixture 5 c.c. of N/1 sulphuric acid, which precipitated the iodine, followed immediately by 5 c.c. of N/1 sodium hydroxide to restore the alkalinity and dissolve the iodine. The reaction was then normal. After standing for 90 minutes the solution was acidified and titrated with N/10 sodium thiosulphate, thus removing the free iodine.

Estimation of Iodoform.—The iodoform was then extracted from the solution by means of ether, the ether extract washed with water, treated with alcoholic potassium hydroxide, and heated on the water bath (15–20 minutes) to hydrolyse the iodoform and convert it into potassium iodide. The iodide was then estimated by VOLHARD'S method and found to be equivalent to 2.44 c.c. of N/10 iodine. A similar experiment in which the iodide was estimated gravimetrically gave a result equivalent to 2.40 c.c. of N/10 iodine.

Estimation of Iodide.—The washings from the ether solution were added to the main aqueous solution and the iodide present estimated by VOLHARD'S method. This was

found to be equivalent to 37.32 c.c. N/10 iodine. This figure, of course, represents the sum of the iodide produced by the action of iodine on kojic acid, together with that produced in the back titration with thiosulphate of the excess of iodine present after the reaction.

This latter figure \equiv 29.71 c.c. N/10 thiosulphate or iodine (mean of duplicate estimations).

Therefore, the iodide produced in the reaction between kojic acid and iodine

$$\equiv 37.32 \text{ c.c.} - 29.71 \text{ c.c.}$$

$$\equiv 7.61 \text{ c.c. N/10 iodine.}$$

The total amount of iodine absorbed by the kojic acid is now given by the difference between the thiosulphate reading in the blank estimation, using water alone,

$$\equiv 39.86 \text{ c.c. N/10 thiosulphate or iodine,}$$

and the thiosulphate reading in the actual estimation, using kojic acid,

$$\equiv 29.71 \text{ c.c. N/10 thiosulphate or iodine.}$$

Therefore, the total iodine absorbed by kojic acid

$$\equiv 39.86 \text{ c.c.} - 29.71 \text{ c.c.}$$

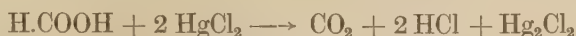
$$\equiv 10.15 \text{ c.c. N/10 iodine.}$$

Thus, these experiments show that 10.02 c.c. M/100 kojic acid absorb 10.15 c.c. N/10 iodine, producing iodoform equivalent to 2.44 c.c. N/10 iodine and iodide equivalent to 7.61 c.c. N/10 iodine. The inference from these figures is that one molecule of kojic acid absorbs 10 atoms of iodine (thus confirming the results previously described) with the production of one molecule of iodoform (this would require a theoretical value of 3.0 c.c. N/10 iodine in place of 2.44 c.c. actually obtained), and 7 molecules of iodide (requiring a theoretical value of 7.0 c.c. N/10 iodine in place of 7.61 c.c. actually obtained experimentally). It is also obvious that no substituted iodine derivatives other than iodoform are produced. The divergence between the experimental and theoretical figures is probably due to the fact that the iodoform undergoes slight decomposition in the alkaline hypoiodite solution with the formation of a corresponding amount of iodide.

(3). *Estimation of Formic Acid.*—The method of estimation finally adopted was based on that described by FINCKE (1913). The details are :—

To 10 c.c. of a M/100 solution of kojic acid were added 120 c.c. of water, and 50 c.c. of the alkaline hypoiodite solution, prepared as described in the estimation of iodoform, followed by 5 c.c. of N/1 sulphuric acid and then 5 c.c. of N/1 sodium hydroxide. The mixture was allowed to stand for 90 minutes, acidified with 5 c.c. of N/1 sulphuric acid and the iodoform and liberated iodine removed by three extractions with ether. To

the extracted solution was added silver carbonate, prepared from 25 c.c. of N/10 silver nitrate solution by the addition of sodium carbonate in slight excess and well washed by decantation. The mixture was well shaken in order to precipitate the whole of the iodine present as silver iodide, which was filtered off, and then 20 c.c. of N/10 hydrochloric acid were added to the filtrate to precipitate the excess silver as silver chloride, which, in its turn, was filtered off. The filtrate was then neutralised to phenolphthalein, evaporated *in vacuo* almost to dryness, the residue dissolved in water, and to this was added a solution containing 2 gm. sodium acetate, 2 gm. mercuric chloride, and 1.5 gm. sodium chloride. After standing overnight in the cold, the solution was filtered, made up to about 100 c.c., and heated for 2 hours in a boiling water bath. The precipitated mercurous chloride was filtered on a Gooch crucible, dried at 100° C. and weighed. From this weight the amount of formic acid was calculated according to the equation



In two experiments the weight of formic acid produced from 10 c.c. of M/100 kojic acid after treatment with iodine was 0.0048 gm. and 0.0044 gm. respectively. Theoretically, if one molecule of formic acid is produced from one molecule of kojic acid, this weight should be 0.0046 gm. Hence it follows that in this reaction one molecule of kojic acid gives rise to one molecule of formic acid.

(4) *Estimation of Oxalic Acid.*—The details of the preparation of the solution containing the products of reaction between kojic acid and iodine, for the estimation of oxalic acid, are the same as those described in the estimation of iodoform, except that ten times the usual quantities of reagents were used throughout and N/1 hydrochloric acid was used in place of N/1 sulphuric acid. Thus 100 c.c. M/100 kojic acid = 0.142 gm. of the acid, were used for the estimation.

When the reaction was complete the solution was acidified with 50 c.c. of N/1 hydrochloric acid and evaporated *in vacuo* to about 100 c.c. By this means the iodoform and liberated iodine were completely removed, and the solution was sufficiently concentrated to allow of the precipitation of the oxalic acid as calcium oxalate. This was carried out in the usual way, after making the solution alkaline with ammonia, and then slightly acid with acetic acid, by the addition of 5 c.c. of 20 per cent. calcium acetate to the boiling solution. The precipitated calcium oxalate was estimated by titration with N/10 permanganate.

In duplicate experiments the oxalic acid present was equivalent to 15.80 c.c. and 15.50 c.c. of N/10 permanganate respectively. Now, using 100 c.c. of M/100 kojic acid solution, for every molecule of oxalic acid produced by one molecule of kojic acid, the amount of N/10 permanganate theoretically required to oxidise the oxalic acid would be 20.0 c.c. Hence it follows that in the reaction between iodine and kojic acid one molecule of the latter gives rise to one molecule of oxalic acid. The somewhat low experimental results obtained may be readily explained by the appreciable solubility

of calcium oxalate in the relatively concentrated solution of salts from which it was precipitated.

(5) *Estimation of Glycollic Acid*.—Since no specific method is available for the estimation of glycollic acid, advantage was taken of the fact that glycollic acid is the only product of the action of iodine on kojic acid which contains a hydroxyl group which can be acetylated by means of acetic anhydride and sodium acetate, to estimate the acid by this means. Preliminary experiments with pure glycollic acid had shown that this reaction provides a reasonably satisfactory quantitative method.

The experimental details (amounts of kojic acid, reagents, etc.) are the same as those used in the preparation of the reaction mixture for the estimation of oxalic acid. When the reaction was complete the solution was acidified with 50 c.c. of N/1 sulphuric acid and the iodine and iodoform extracted with ether. The iodide was precipitated by means of silver carbonate, the excess silver removed by hydrochloric acid, the solution filtered, neutralised and evaporated to low bulk *in vacuo*. The residual solution was washed into a 250 c.c. round-bottomed flask and evaporated to dryness in a gentle stream of hot air. The dry residue was then acetylated by means of acetic anhydride and sodium acetate in the manner of a glycerol analysis. After heating for one hour under a reflux the acetylation product was dissolved in cold water, and exactly neutralised with N/1 sodium hydroxide; 50 c.c. of N/1 sodium hydroxide solution were added from a pipette, the solution boiled under a reflux for fifteen minutes, cooled, and the excess of alkali titrated with acid. The alkali used up in hydrolysis is equivalent to the hydroxyl groups present, and was in this estimation

$$\equiv 0.78 \text{ c.c. N/1 NaOH.}$$

Since it is evident by calculation that, for every hydroxyl group produced from one molecule of kojic acid, with the quantities of kojic acid used in this estimation, 1.0 c.c. of N/1 sodium hydroxide would be used up in hydrolysis, it is clear that in the action of iodine on kojic acid one molecule of kojic acid gives rise to a substance containing one hydroxyl group, *i.e.*, to one molecule of glycollic acid.

(6) *Estimation of Total Acidity*.—The total acidity was estimated by titrating, with standard sodium hydroxide solution, the acid produced during the reaction between iodine and kojic acid, correction being made for acidity of reagents by carrying out a parallel blank experiment in which water was used in place of kojic acid solution. This procedure is rendered possible, since no acidity is produced during the titration of the residual iodine with sodium thiosulphate. The details of the estimation are:—

To 10.02 c.c. of M/100 kojic acid solution were added 40 c.c. of N/10 iodine solution and 50 c.c. of N/10 sodium hydroxide, and the mixture allowed to stand for 90 minutes. A parallel blank experiment, with 10 c.c. of water in place of the kojic acid solution, was carried out in exactly the same way. To each was now added 60 c.c. of N/10 hydrochloric acid solution to ensure an excess of acid, and the residual

iodine titrated back with N/10 thiosulphate. The whole volume of thiosulphate solution used was :—

| | | | | | | | |
|-------------------------------|----|----|----|----|----|----|------------|
| Blank experiment | .. | .. | .. | .. | .. | .. | 39.95 c.c. |
| Kojic acid experiment | .. | .. | .. | .. | .. | .. | 30.03 „ |
| Iodine absorbed by kojic acid | .. | .. | .. | .. | .. | .. | 9.92 c.c. |

The two solutions were then titrated with N/10 sodium hydroxide, using phenolphthalein as indicator. The volume of sodium hydroxide used was :—

| | | | | | |
|--|----|----|----|----|-----------------------|
| Kojic acid experiment | .. | .. | .. | .. | 20.83 c.c. N/10 NaOH. |
| Blank experiment | .. | .. | .. | .. | 9.73 „ „ „ |
| Total acidity produced during the reaction between iodine and kojic acid | .. | .. | .. | .. | 11.10 „ |

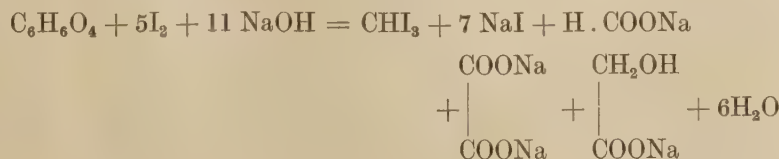
Thus, since 10.02 c.c. of M/100 kojic acid absorb 9.92 c.c. of N/10 iodine and produce acid equivalent to 11.10 c.c. of N/10 sodium hydroxide, it follows that during the reaction between iodine and kojic acid, one molecule of the latter absorbs 10 atoms of iodine (thus again confirming the previous results), and produces acid equivalent to 11 molecules of a monobasic acid. This is distributed as follows :—

- (a) 7 molecules as hydriodic acid equivalent to the 7 molecules of alkaline iodide estimated (see p. 143).
- (b) 1 molecule as formic acid (see p. 144).
- (c) 2 molecules as one molecule of oxalic acid (see p. 145).
- (d) 1 molecule as glycollic acid (see p. 146).

Hence it is clear that no other acid is formed during the reaction, and this is supported by the fact that, although carbon dioxide was tested for among the reaction products, its presence could not be demonstrated.

It is now possible, from a consideration of the results obtained, to construct an equation for the reaction. The results may be briefly summarized. It has been proved that one molecule of kojic acid in alkaline solution takes up 10 atoms of iodine (p. 140), and gives rise to one molecule of iodoform and 7 molecules of iodide (p. 143), one molecule of formic acid (p. 144), one molecule of oxalic acid (p. 145), one molecule of glycollic acid (p. 146), and a total acidity equivalent to 11 molecules of monobasic acid (p. 146).

Thus the equation for the action of iodine on kojic acid in alkaline solution is



B.—*Mechanism of the Reaction between Iodine and Kojic Acid.*

In considering the mechanism of the reaction between alkaline iodine solution and kojic acid it is well to bear in mind the fact that alkaline iodine solution can exercise a double function. On the one hand, it may act as an alkaline hydrolytic agent, and, on the other, it may act as an oxidising agent. Hence the final products of the reaction between alkaline iodine and kojic acid, *i.e.*, formic, glycollic, and oxalic acids, may possibly arise as a result either of hydrolysis, or of oxidation, or of a combination of the two processes. Iodoform must, of course, arise by a process of substitution from some intermediate compound.

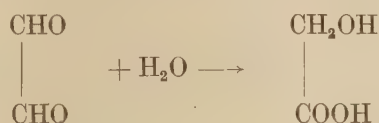
Let us consider these actions of hydrolysis and oxidation separately.

(1) *Hydrolysis*.—No information is available as to the products of hydrolysis of kojic acid by means of sodium hydroxide, but a close analogy may be found in the action of barium hydroxide on the dimethyl ether of kojic acid. YABUTA (1916) investigated this reaction, and found that the products of hydrolysis were equimolecular proportions of (1) methoxyacetone, (2) formic acid, and (3) methoxyacetic acid. If the same type of hydrolysis takes place as a result of the action of sodium hydroxide on kojic acid the products of hydrolysis should be (1) hydroxyacetone or pyruvic alcohol, (2) formic acid and (3) glycollic acid or some substance which gives rise to glycollic acid on hydrolysis.

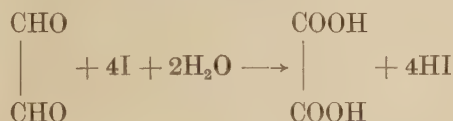
(2) *Oxidation*.—Since formic and glycollic acids have already been shown to be formed as a result of the action of alkaline iodine on kojic acid, it was obviously desirable to investigate the products of the reaction between alkaline iodine and pyruvic alcohol. This investigation, details of which are given later (p. 150), showed that pyruvic alcohol is acted on by alkaline iodine, one molecule of the alcohol absorbing 6 atoms of iodine and giving rise to iodoform, glycollic acid, and hydriodic acid, but not to either formic or oxalic acids. Hence these two acids must arise from the other half of the kojic acid molecule. Referring again to YABUTA's results it is seen that formic acid, but not oxalic acid, is a product of the hydrolysis of the dimethyl ether of kojic acid with barium hydroxide. Hence, it seems reasonable to assume that formic acid is also a product of hydrolysis in the case of kojic acid, and it only remains now to obtain a satisfactory explanation for the production of oxalic acid.

Up to the present only six atoms of iodine have been accounted for, *i.e.*, in the oxidation of one molecule of pyruvic alcohol. Hence, of the ten atoms of iodine shown in the equation, four remain to be considered, and it does not seem unreasonable to conclude that these are used up in an oxidative process resulting in the production of oxalic acid. It seems obvious at first sight that the oxalic acid arises by the oxidation of the glycollic acid, which, by analogy with YABUTA's results, should be formed along with formic acid and pyruvic alcohol. But if this were so, why should the same oxidising agent, which is thus supposed to oxidize glycollic acid to oxalic acid, not only produce glycollic acid and not oxalic acid from pyruvic alcohol, but also have no oxidising action itself on glycollic acid as was proved by direct experiment? The idea that the oxalic acid arises by oxidation of glycollic acid must, therefore, be abandoned.

It was previously pointed out that, if one may argue by analogy with YABUTA's results, kojic acid should give rise on hydrolysis to pyruvic alcohol, formic acid, and glycollic acid or some substance which gives rise to glycollic acid on hydrolysis. Hence a reasonable explanation of the origin of the oxalic acid seems to be that during the action of alkaline iodine on kojic acid, in addition to pyruvic alcohol and formic acid, a two carbon compound is momentarily formed which may give rise, on the one hand, by hydrolysis, to glycollic acid, or, on the other, by oxidation, to oxalic acid. Such a compound is glyoxal $\text{CHO} \cdot \text{CHO}$. Glyoxal, even in the cold, is converted by alkalies, by a CANNIZZARO reaction, into glycollic acid.

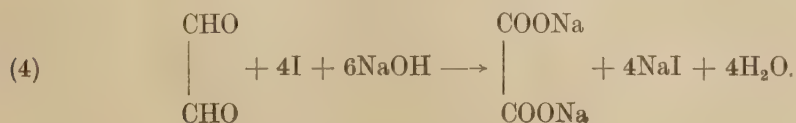
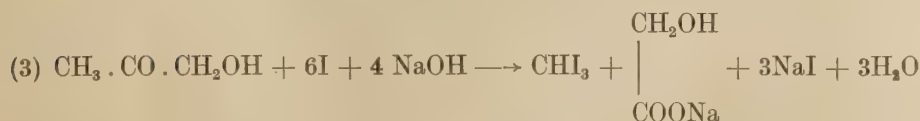
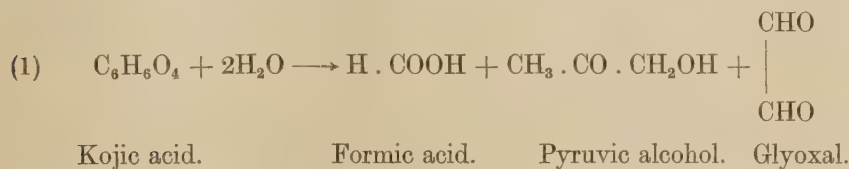


and is oxidized by alkaline iodine to oxalic acid according to the equation

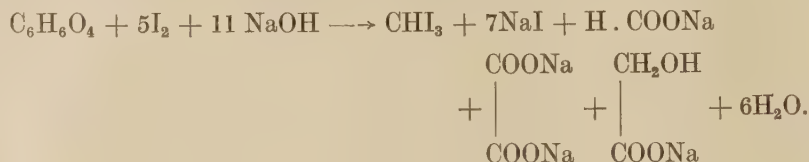


It seems, then, that in the presence of a large excess of alkaline iodine solution, oxidation of the momentarily formed glyoxal takes place in preference to hydrolysis, and hence, oxalic acid is the final product, and not glycollic acid, as would be expected if the action of alkaline iodine on kojic acid followed strictly the reaction occurring during the hydrolysis of the dimethyl ether of kojic acid by barium hydroxide. Glycollic acid is indeed a product of the reaction between alkaline iodine and kojic acid, but it is evident that it arises from the pyruvic alcohol and not from the compound (presumably glyoxal) corresponding to the precursor of the methoxyacetic acid found by YABUTA.

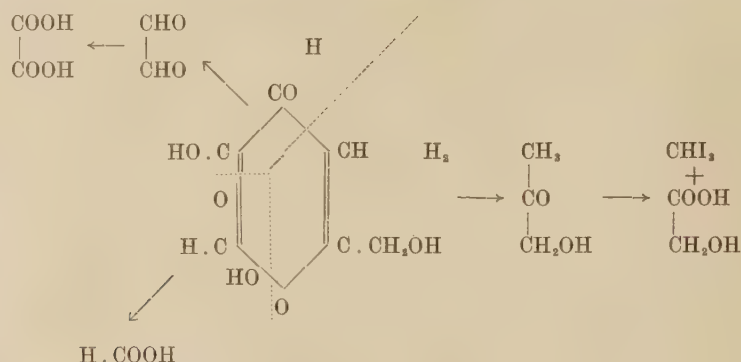
The course of the reaction may therefore be summed up as follows :—



Substituting equations (2), (3), and (4) in equation (1) we get the equation previously given on p. 147.



The mechanism of the breakdown of the kojic acid may be represented graphically as follows:—



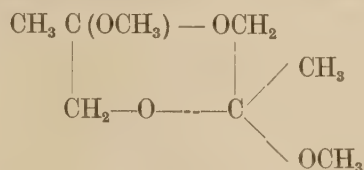
The Action of Alkaline Iodine on Pyruvic Alcohol.—Pyruvic alcohol was prepared from monobromoacetone by NEF's method (1904) and subjected to the action of alkaline iodine under the same conditions as in the kojic acid reaction. Very variable results were obtained, depending on the amount of pyruvic alcohol employed, but the relative amount of iodine used was found to increase as the quantity of pyruvic alcohol was cut down, until, finally, a maximum and constant value was attained when the iodine was present in quantity amounting to about twenty times that theoretically required.

A stock solution of pyruvic alcohol was made up containing 0.5993 gm. per litre, and, to varying amounts of this, indicated in column 1 of the following table, 40 c.c. of N/10 iodine and 50 c.c. of N/10 sodium hydroxide were added in each case, exactly as was done with kojic acid. The amount of N/10 iodine used by the pyruvic alcohol and calculated in terms of c.c. N/10 iodine per 1 c.c. M/10 pyruvic alcohol is shown below.

| Cubic centimetres of stock solution of pyruvic alcohol. | Cubic centimetres N/10 iodine equivalent to 1 c.c. M/10 pyruvic alcohol. |
|---|---|
| 10 | 3.4 |
| 5 | 4.9 |
| 2 | 6.5 |
| 2 | 6.0 |
| 1 | 6.3 |
| 0.5 | 5.9 |

These figures indicate that, provided a sufficient excess of alkaline iodine is used, an approximately constant absorption value is obtained, corresponding to 6 atoms of iodine per molecule of pyruvic alcohol.

As pyruvic alcohol itself is rather unstable, it was considered advisable to confirm these results by utilising the rather curious compound, bis-pyruvic alcohol methyl alcoholate,



as a source of pure pyruvic alcohol. Bis-pyruvic alcohol methyl alcoholate is readily prepared from pyruvic alcohol and methyl alcohol (NEF, 1904) and has the great advantages as a standard for the purpose in view that it crystallises well, is quite stable, but is very easily hydrolysed by acids to give pyruvic alcohol and methyl alcohol.

A standard solution of the recrystallised alcoholate was therefore made up (M/200 equivalent to M/100 pyruvic alcohol) and aliquot portions were hydrolysed before use by heating at 60° C. in a closed flask for one hour with 10 c.c. of N/1 hydrochloric acid. After cooling, the acid was neutralised by the addition of 10 c.c. of N/1 sodium hydroxide, and N/10 iodine and alkali were added as with pyruvic alcohol. As before, a constant value for the iodine used up was not attained until the iodine employed was in very great excess of the theoretical.

| Cubic centimetres of stock solution of alcoholate. | Cubic centimetres N/10 iodine equivalent to 1 c.c. M/10 pyruvic alcohol. |
|--|---|
| 5 | 4.7 |
| 2 | 5.5 |
| 1 | 6.2 |
| 1 | 6.1 |
| 0.5 | 6.0 |
| 0.25 | 6.0 |

These results confirm those previously obtained and indicate that one molecule of pyruvic alcohol absorbs 6 atoms of iodine, behaving in this respect like acetone. The products formed during this reaction and the equation representing it were investigated as follows:—

0.9 gm. of bis-pyruvic alcohol methyl alcoholate was hydrolysed by heating for one hour at 60° C. with 20 c.c. of N/1 sulphuric acid. The mixture was then diluted with water to about 2700 c.c. and 25.4 gm. of iodine dissolved in 150 c.c. of pure methyl alcohol were added, followed by a solution of 43 gm. of crystalline barium hydroxide

dissolved in warm water. The final volume was about 3 litres. After standing two hours, the mixture was investigated qualitatively by the same method as that described on p. 142 for the investigation of the reaction products of kojic acid and alkaline iodine.

The following compounds were tested for

| | |
|-------------------------|---------|
| (1) iodoform | present |
| (2) formic acid | absent |
| (3) oxalic acid | absent |
| (4) glycollic acid..... | present |

and the last named was isolated as the calcium salt which gave the following analysis:—

| | Found. | Calculated for $\text{Ca}(\text{C}_2\text{H}_3\text{O}_3)_2 \cdot 4\text{H}_2\text{O}$ |
|---------------------------|-----------------|--|
| Water of crystallisation. | 27·52 per cent. | 27·48 per cent. |
| | | Calculated for $\text{Ca}(\text{C}_2\text{H}_3\text{O}_3)_2$ |
| Carbon in anhydrous salt. | 25·77 per cent. | 25·26 per cent. |

The equation for the reaction between pyruvic alcohol and alkaline iodine is similar to that for the reaction with acetone except that glycollic acid is formed in place of acetic acid. It is therefore,



Summary.

A method is described (p. 140) for the quantitative estimation of kojic acid (5-hydroxy-2-hydroxymethyl- γ -pyrone) which depends on the fact that kojic acid is quantitatively decomposed by alkaline iodine solution under experimental conditions which have been determined and of which details are given.

The equation of this reaction has been established (p. 141) and the mechanism by which the reaction takes place has been determined (p. 148).

Studies in the Biochemistry of Micro-organisms.

PART IX.—*On the production of Mannitol from Glucose by Species of Aspergillus.*

By JOHN HOWARD BIRKINSHAW, JOHN HENRY VICTOR CHARLES, ARTHUR CLEMENT
HETHERINGTON and HAROLD RAISTRICK.

Mannitol ($C_6H_{14}O_6$) is the most common of the naturally occurring hexahydric alcohols. It is a curious fact that while glucose is the most commonly occurring hexose, the corresponding alcohol, sorbitol, is of relatively uncommon occurrence, while mannose and fructose, which are structurally related to mannitol, are not of such common occurrence as glucose.

Mannitol is a common constituent of many plants, but occurs principally in the curious plant exudation known as "manna," and is at present produced technically by two methods, both of which are open to obvious disadvantages.

(a) From "manna."

(b) By the bacterial fermentation of fructose.

(a) *Preparation from manna.*—Manna is a crystalline exudation occurring on various plants, but principally on the flowering or manna ash tree (*Fraxinus ornus*). The separation of mannitol from manna is a relatively easy process, only involving purification by fractional crystallisation. The product obtained is of good quality, but this method of supply is open to the serious objection that the quantity of manna available fluctuates very considerably during different seasons, and is largely dependent on weather conditions. Thus, in a wet season much of the manna produced is washed away on account of its ready solubility in water.

(b) *By the bacterial fermentation of fructose.*—For résumé and bibliography see STILES, PETERSON and FRED (1925).

It has long been known that mannitol is a metabolic product of certain bacteria grown on sugar solutions. The mannitic fermentation of wine has been intensively studied, and in recent years a process has been devised for the production of mannitol by bacterial fermentation. This method of preparation is open to two objections.

(1) The bacteria used convert only fructose into mannitol, and while they also act on glucose no mannitol is produced from this sugar, its place being taken by other decomposition products, chiefly lactic acid. Fructose is too expensive to use as a raw material for a fermentation process, and if sucrose, which gives rise to fructose

and glucose on hydrolysis, is used, the yield of mannitol is, of course, only half that produced from pure fructose. This, however, is not the most serious objection to the use of sucrose; its worst feature is the production of large quantities of soluble non-volatile by-products from the glucose part of the sucrose molecule. The main product arising from glucose, and equal in amount to the yield of mannitol from fructose, is lactic acid, which, either as the free acid or as the calcium salt, interferes very seriously with the recovery of mannitol from the fermentation solutions.

(2) Another objection to this method is the difficulty of recovering mannitol from the fermented liquors. This difficulty arises in part, as described in the last paragraph, from the production of calcium lactate, but is further intensified by the fact that the bacteria used for the fermentation require a source of organic nitrogen for growth, *e.g.*, proteins or peptones. These are not only expensive, and so contribute in part to the economic objections to this method, but they also add very considerably to the recovery difficulties.

During the course of investigations on the metabolic products of various common moulds it has been found here that mannitol is a fairly common product of the fermentation of glucose by numerous fungi. Since these micro-organisms grow on very simple media, requiring only inorganic and not organic nitrogen for their nutrition, and since they produce mannitol either from glucose or fructose, it was felt that the main objections to the above bacteriological method of producing mannitol might be overcome by using moulds as the active agents. Actual practice has indeed shown that the mannitol produced is very easily isolated from mould fermentation solutions.

The work carried out on the production of mannitol by various selected species of fungi is described in this paper.

It was pointed out as early as 1813 by VAUQUELIN and by BRACONNOT that mannitol is found in the *tissues* of the higher fungi. This has since been confirmed by a number of workers, who have shown that in a large variety of the agarics mannitol forms the chief part of the non-nitrogenous reserve material. More recently various observers have reported its occurrence in the mycelium of some of the lower fungi, particularly in different species of *Aspergillus* and *Penicillium*. BOURQUELOT (1889-90), working with *Aspergillus niger*, showed that mannitol and trehalose are present in the mycelium of this mould, and that the relative proportions of these two bodies vary in an interesting manner with different stages of growth.

All these observations, however, relate to the isolation and identification of mannitol in the *tissues* of the fungus investigated. It appears to be regarded as a reserve food material for the growing organism, comparable with the glycogen of yeast, rather than as a definite fermentation product, and no attempt seems to have been made to study it from the latter standpoint. In the course of the work about to be described it was shown that the metabolism solutions of certain species of *Aspergillus* contained large amounts of mannitol; yields up to 50 per cent. of the original glucose were obtained,

thus putting beyond doubt the view that mannitol is a genuine product of the fermentation of this sugar by moulds.

As a result of the systematic examination of various species of fungi, involving the preparation of carbon balance sheets for each species examined, as described in Parts II and III, it was found that certain species of *Aspergillus* showed in the metabolism solution a comparatively large amount of carbon which was not accounted for in any of the estimated products. This portion of the balance sheet, which was called "carbon unaccounted for," was in some cases very considerable, and with some species has been shown to consist almost entirely of mannitol. The history of these species on which work is described in this paper will now be given. They fall into three groups:—

- (1) White species of *Aspergillus*.
- (2) *Aspergillus elegans*.
- (3) Species of *Aspergillus nidulans*.

Group 1. White species of *Aspergillus*.

- (1) *Aspergillus* sp. THOM 4640.490, Catalogue No. Ac. 56. Obtained from Dr. CHARLES THOM, of the U.S.A. Bureau of Agriculture, Washington.
- (2) *Aspergillus* sp. THOM 4640.489, Catalogue No. Ac. 55. Obtained from Dr. THOM.
- (3) *Aspergillus* sp. Catalogue No. Ac. 10. Isolated in the laboratory at Ardeer, from a bench contamination of CZAPEK-DOX agar.

Group 2. *Aspergillus elegans*.

- (4) *Aspergillus elegans* GASPARINI, Catalogue No. Ac. 40. Purchased from the Centraalbureau voor Schimmelcultures at Baarn.

Group 3. Species of *Aspergillus nidulans*.

- (5) *Aspergillus nidulans* EIDAM, Catalogue No. Ac. 67. Purchased from the Centraalbureau voor Schimmelcultures at Baarn.
- (6) *Aspergillus nidulans*, Catalogue No. Ac. 78. Purchased from PRIBŘAM, Vienna, and as received bore the label, *A. nidulans* (HANN).
- (7) *Aspergillus nidulans* var. *Nicollei* PINOY, Catalogue No. Ac. 85. Purchased from the Centraalbureau voor Schimmelcultures at Baarn.
- (8) *Aspergillus nidulans* 110 (ascosporic), Catalogue No. Ac. 79. Purchased from the British National Collection of Type Cultures via Miss CHURCH, of the U.S.A. Department of Agriculture, Washington.
- (9) *Aspergillus nidulans*, Catalogue No. Ac. 98. Obtained from Mr. F. T. BROOKS, of Cambridge, and identified by Miss CHURCH.

The abridged balance sheets for these different species, as originally prepared in the metabolism experiments, are collected together in Table I for convenience of comparison.

Examination of these carbon balance sheets shows that, with the exception of the item "carbon as volatile neutral compounds," no other products are formed in appreciable amounts except those included under the heading "carbon unaccounted for." This has considerable practical importance, since, as the volatile neutral compounds can easily be removed by distillation, it follows that the only residual products in solution will be those under the heading of "carbon unaccounted for," so that it also follows that, if this item is shown to consist entirely or principally of mannitol, the recovery of this material must of necessity be a relatively simple matter.

Quantitative Estimation of Mannitol produced by Different Species of Aspergillus.

A number of the above species have been examined *quantitatively* from the point of view of mannitol production. The method adopted was the following:—

A CZAPEK-DOX solution containing 5 per cent. of glucose was distributed in 750 c.c. flasks, each flask containing 250 c.c. of medium. A number of these were then sown with spores of the particular species under investigation, and the necks of the flasks fitted with rubber connections, as described in Part II, p. 15. The flasks were incubated at 24° C., and each day about 300 c.c. of sterile air were passed through each flask during the course of half an hour. The flasks were then shut off from the air supply and no further air passed through until the following day. At the end of the incubation period the contents of the flask were filtered, and filtrate and washings were neutralised with N/1 sodium hydroxide solution to p_H 7, made up to a known volume, and a carbon balance sheet prepared on a portion of this solution exactly as described in Part II. The remainder of the metabolism solution was evaporated *in vacuo* and the distillate examined for alcohol. In all those cases where an appreciable amount of "carbon as volatile neutral compounds" occurs in the balance sheet, this was shown to consist almost entirely of ethyl alcohol. In those cases where it was considered advisable to do so, the residual glucose was then removed by fermentation with a pure culture of yeast. Whether this procedure was followed or not, the next step was to remove all precipitable material by treatment with normal and basic lead acetate. The filtrate was freed from lead by means of hydrogen sulphide, the solution filtered, and evaporated *in vacuo* to low volume, and then made up to a known volume for analysis.

In a portion of this solution the mannitol was estimated by treatment with borax and polarisation, as described in Part X. In another portion the mannitol was estimated by determining the acetyl value by treatment with acetic anhydride and anhydrous sodium acetate, as described in Part X. The results of these estimations are given in Table II.

TABLE II.—Analyses of Metabolism Solutions.

| Experimental details. | <i>A. Sp.</i> THOM 4640.490 | <i>A. Sp.</i> THOM 4640.489 (p_H of medium 4.2). | <i>A. nidu-</i> <i>lans.</i> EIDAM. | <i>A. nidu-</i> <i>lans.</i> | <i>A. nidu-</i> <i>lans</i> var. <i>Nicollei</i> PINOY. | <i>A. nidu-</i> <i>lans</i> (asco- sporic). | <i>A. nidu-</i> <i>lans.</i> |
|--|-----------------------------------|--|---|---------------------------------|--|---|---------------------------------|
| Catalogue number | Ac. 56 | Ac. 55 | Ac. 67 | Ac. 78 | Ac. 85 | Ac. 79 | Ac. 98 |
| Experiment number | P 31 | P 18 | P 10 | P 9 | P 17 | P 8 | P 25 |
| Incubation period in days | 62 | 77 | 39 | 40 | 34 | 59 | 46 |
| Total carbon in solution | 2.819 | 2.325 | 1.633 | 1.505 | 1.880 | 2.509 | 2.349 |
| Carbon as residual glucose | 0.728 | 0.067 | 0.128 | 0.102 | 0.356 | 1.505 | 1.335 |
| Carbon as CO ₂ in solution... .. | 0.021 | 0.004 | 0.033 | 0.022 | 0.015 | 0.008 | 0.021 |
| Carbon as volatile acids | 0.046 | 0.054 | 0.014 | 0.021 | 0.016 | 0.005 | 0.015 |
| Carbon as non-volatile acids | 0.129 | 0.144 | 0.131 | 0.109 | 0.109 | 0.094 | 0.113 |
| Carbon as volatile neutral com- pounds | 0.079 | 0.216 | 0.777 | 0.707 | 0.679 | 0.026 | 0.012 |
| Carbon as synthetic compounds | 0.034 | 0.047 | 0.035 | 0.053 | 0.053 | 0.078 | 0.069 |
| Carbon unaccounted for (by differ- ence) | 1.782 | 1.793 | 0.515 | 0.491 | 0.652 | 0.793 | 0.784 |
| Carbon as mannitol by polarimeter | 1.131 | 1.669 | 0.388 | 0.397 | 0.494 | — | 0.644 |
| Carbon as mannitol from acetyl value | 1.124 | 1.768 | 0.449 | 0.451 | 0.580 | — | 0.729 |

N.B.—In the above table the figures given for different classes of carbon represent gm. carbon per 250 c.c. original medium. Since the medium contained 5 per cent. of glucose, *i.e.*, a total of 12.5 gm. glucose, which is equivalent to 5 gm. of carbon as glucose, multiplication of any of the above figures by 20 gives an approximate percentage yield of the particular product.

Isolation of Mannitol from the Metabolism Solutions of different Fungi.

In the results given in Table II mannitol was estimated but was not isolated. In the experiments described below, mannitol was isolated and identified, and a rough quantitative idea formed as to the yields obtained. It is interesting to note that in those cases where both a quantitative estimation and an isolation experiment have been carried out, the results agree quite reasonably well.

Before passing on to a detailed description of these experiments, note will be made of a difficulty which arose at the beginning of this work and which was not overcome for some time. It was found that while the quantitative results obtained in the metabolism experiments could be reproduced consistently, the yield of product was disappointingly small when an attempt was made to prepare larger quantities of fermentation solutions for investigation. The only difference between the conditions of experiment was that, whereas in the metabolism experiments each flask had a limited and known volume of air passed through it, in the earlier "bulk" experiments this method of aeration was altered, and replaced by plugging each of the flasks with an

ordinary cotton-wool plug. Hence, while in the metabolism experiments the aeration was limited, in the larger scale experiments it was unrestricted. The difference in results obtained will be best appreciated by comparing the salient features of two carbon balance sheets prepared for *Aspergillus elegans* (Ac. 40) grown under these two conditions.

It is obvious that unrestricted aeration prevents the accumulation either of alcohol ("volatile neutral compounds") or of the "carbon unaccounted for."

The production of mannitol by different species will now be dealt with under each species investigated.

(1) *Aspergillus elegans* (Ac. 40).

(a) *With unrestricted aeration.*—Five litres of a CZAPEK-DOX glucose solution were made up and distributed in 350 c.c. lots in 1 litre flasks plugged with cotton wool. These were sterilized, sown with *Aspergillus elegans*, and incubated for 37 days at 23° C. At the end of this period the metabolism solution was filtered, and a carbon balance sheet prepared on a portion of it. This is given in Table III, column 3. The remainder

TABLE III.—Comparison of Carbon Balance-Sheets, Limited and Unrestricted Aeration.

| | | | | | | | Ac. 40 grown under conditions of aeration as in metabolism experi- ments, i.e., limited aeration. | Ac. 40 grown in flasks plugged with cotton wool, i.e., unrestricted aeration. |
|--|-----|-----|-----|-----|-----|-----|--|--|
| Incubation period in days | ... | ... | ... | ... | ... | ... | 48 | 37 |
| Total carbon in solution | ... | ... | ... | ... | ... | ... | 2.420 | 1.536 |
| Carbon as residual glucose | ... | ... | ... | ... | ... | ... | 0.658 | 0.969 |
| Carbon as volatile neutral compounds | ... | ... | ... | ... | ... | ... | 0.569 | 0.021 |
| Carbon unaccounted for (by difference) | ... | ... | ... | ... | ... | ... | 0.922 | 0.297 |

Carbon as different compounds all expressed as gm. carbon per 250 c.c. medium

of the solution was evaporated *in vacuo* to a thick syrup, extracted with hot alcohol, and allowed to stand overnight. The clear liquid was poured off, evaporated to a syrup, and re-extracted with absolute alcohol. Evaporation of the alcoholic extract gave rise to well-defined rhombic prisms which proved to be a compound of glucose with sodium chloride, $2C_6H_{12}O_6 \cdot NaCl \cdot H_2O$. As it was found impossible to isolate anything other than glucose, the residues were freed from alcohol, dissolved in water, and the glucose removed by fermentation with yeast. The glucose-free residue was evaporated to a syrup, and the syrup extracted with boiling alcohol. On standing, the alcoholic solution deposited clusters of needles, which were filtered off and dried.

On heating, these crystals showed signs of softening at 152° C., and melted completely

at 160°–162° C. On mixing with pure mannitol (melting point 163°–164° C.) they melted at 163° C. This indicated that the crystals consisted of impure mannitol. They were further characterized by conversion into a benzal derivative, using 0.1 gm. of the crude crystals, according to the method of FISCHER and FAY for *l*-iditol (1895). On shaking for a few minutes, after the addition of 0.2 c.c. concentrated hydrochloric acid and 0.2 c.c. benzaldehyde, the contents of the tube solidified. The mixture was allowed to stand overnight, then water was added and the solid filtered off and washed two or three times with alcohol, and finally with ether. After this treatment, perfectly colourless crystals were obtained melting at 215°–217° C. FISCHER gives the melting point of mannitol tribenzoylacetal as 215°–217° C.

It is thus evident that mannitol is one of the products of the growth of *Aspergillus elegans* on glucose, even under conditions of unrestricted aeration, although under these conditions the yield of mannitol is very small.

(b) *With restricted aeration*.—Three litres of the usual 5 per cent. glucose CZAPEK-DOX medium were distributed between twelve 750 c.c. flasks. After sterilising and sowing with Ac. 40, each flask was fitted with a rubber bung and aeration tubes, as in the metabolism experiments. The flasks were aerated for half an hour each day, and the rate at which the air was supplied was, as far as possible, uniform and comparable with the rate in the quantitative experiments. The flasks were incubated at 23° C. for 48 days, and at the end of this time a carbon balance sheet was prepared on a portion of the mixed, neutralised, and filtered metabolism solution. This is given in Table III, column 2. The remainder of the filtrate was evaporated *in vacuo* to low bulk and immediately crystallised on cooling. Treatment of the distillate will be dealt with later. The crystalline residue was diluted somewhat with water, treated with a considerable quantity of hot, absolute alcohol, and set aside to crystallise. 12 gm. of almost white crystals, having the typical appearance of mannitol, separated, and further quantities were obtained from the mother liquors. Assuming that all the “carbon unaccounted for” is due to mannitol, the yield should be 24.7 gm., and the amount obtained indicates that if allowance is made for the solubility of mannitol, this compound must constitute the greater part of the “carbon unaccounted for.”

A sample of the recrystallised product melted at 163°–164° C. and there was no lowering of the melting point when it was mixed with a sample of pure mannitol. It gave the following results on combustion:—

| | | | | Observed. | Calculated. |
|----------|----|----|----|-----------|-------------|
| Carbon | .. | .. | .. | 39.73 | 39.54 |
| Hydrogen | .. | .. | .. | 7.57 | 7.75 |

The distillate obtained on evaporation of the metabolism solution was fractionated three times. The final fractions consisted of 1–2 c.c. collected below 78° C., and 10–12 c.c. collected from 78°–78.5° C. Both fractions gave the usual qualitative

tests for ethyl alcohol, and neither fraction gave any reaction for aldehydes or ketones when tested with *p*-nitrophenylhydrazine.

(2) *Aspergillus* species (Ac. 10).

(a) *With unrestricted aeration*.—The effect of unrestricted aeration in preventing the accumulation of the “carbon unaccounted for,” as observed with Ac. 40, was also seen in experiments carried out with Ac. 10.

Two flasks of CZAPEK-DOX 5 per cent. glucose were sown with Ac. 10, and incubated at 23° C., with cotton wool plugs in the necks of the flasks. The salient features of the carbon balance sheet on the mixed metabolism solutions from these two flasks are given in Table IV, column 3. In column 2 of the same table, corresponding values are given for comparison for the same mould grown under conditions of restricted aeration.

TABLE IV.—Results of metabolism experiments with Ac. 10.

| | Ac. 10 grown under conditions of aeration as in metabolism experiment, <i>i.e.</i> , restricted aeration. | Ac. 10 grown in flasks plugged with cotton wool, <i>i.e.</i> , unrestricted aeration. |
|---|---|---|
| Incubation period in days | 51 | 35 |
| Total carbon in solution | 2.380 | 2.836 |
| Carbon as residual glucose | 0.802 | 2.238 |
| Carbon as volatile neutral compounds | 0.012 | 0.013 |
| Carbon unaccounted for (by difference) | 1.181 | 0.251 |

Carbon as different compounds all expressed as gm. carbon per 250 c.c. medium.

A small amount of mannitol was isolated from the evaporated metabolism solution from these flasks.

(3) *Aspergillus* species (Ac. 55).

(a) *With varying degrees of aeration*.—The effect of varying degrees of aeration on the growth of Ac. 55 and on the yield of water-soluble metabolic products was investigated in some detail. Since experience showed that the chief water-soluble metabolic product of Ac. 55 is mannitol, complete carbon balance sheets were not prepared in this series of experiments, but a measure of the amount of mannitol produced was obtained by estimating the *total* carbon present in solution and subtracting from this the carbon as residual glucose (Table V, column 6).

The experiment was carried out as follows: A number of 1 litre conical flasks, each containing 350 c.c. of the usual CZAPEK-DOX 5 per cent. glucose solution, were sown

with Ac. 55. Some of the flasks were fitted with sterile rubber bungs and side tubes, as in the metabolism experiments and measured amounts of sterile air, varying from 50 c.c. to 1,000 c.c. per day, were passed through the appropriate flasks daily during the incubation period. The remainder of the flasks were loosely plugged with cotton wool and were left undisturbed until taken off for analysis.

The results obtained are given in Table V, in which all weights are expressed as gm. per 250 c.c. of medium.

TABLE V.—Effect of aeration on yield of metabolic products of Ac. 55.

| Degree of aeration. | Incubation period in days. | Weight of mycelium. | Carbon in glucose. | Total carbon in solution. | Difference, i.e., carbon as metabolic products in solution. | Percentage yield of metabolic products calculated on glucose consumed. |
|--|----------------------------|---------------------|--------------------|---------------------------|---|--|
| | | gm. | gm. | gm. | gm. | |
| 50 c.c. ... | 46 | 0.28 | 3.874 | 4.527 | 0.653 | 58.0 |
| 50 c.c. ... | 66 | 0.31 | 3.092 | 4.274 | 1.182 | 62.0 |
| 100 c.c. ... | 46 | 0.49 | 3.492 | 4.285 | 0.793 | 52.6 |
| 100 c.c. ... | 65 | 0.69 | 2.426 | 3.838 | 1.412 | 54.9 |
| 200 c.c. ... | 46 | 0.76 | 2.830 | 3.893 | 1.063 | 49.0 |
| 200 c.c. ... | 65 | 1.08 | 1.460 | 3.281 | 1.821 | 51.4 |
| 300 c.c. ... | 47 | 0.96 | 2.276 | 3.631 | 1.355 | 49.7 |
| 300 c.c. ... | 64 | 1.24 | 0.910 | 2.936 | 2.026 | 49.5 |
| 400 c.c. ... | 47 | 1.14 | 1.778 | 3.290 | 1.512 | 46.9 |
| 400 c.c. ... | 64 | 1.46 | 0.264 | 2.470 | 2.206 | 46.6 |
| 600 c.c. ... | 48 | 1.37 | 1.731 | 3.082 | 1.351 | 41.3 |
| 600 c.c. ... | 63 | 1.76 | 0.142 | 2.075 | 1.933 | 39.8 |
| 800 c.c. ... | 48 | 1.53 | 1.656 | 3.035 | 1.378 | 41.2 |
| 800 c.c. ... | 63 | 1.93 | 0.112 | 1.835 | 1.723 | 35.3 |
| 1,000 c.c. ... | 49 | 1.88 | 0.727 | 2.411 | 1.683 | 39.4 |
| 1,000 c.c. ... | 62 | 1.97 | 0.090 | 1.585 | 1.495 | 30.4 |
| Unrestricted aeration. Cotton wool plugs | 16 | 0.56 | 4.032 | 4.364 | 0.332 | 34.3 |
| | 19 | 0.64 | 3.896 | 4.295 | 0.399 | 36.2 |
| | 23 | 1.00 | 3.074 | 3.833 | 0.759 | 39.4 |
| | 26 | 0.90 | 3.178 | 3.930 | 0.752 | 41.3 |
| | 34 | 1.90 | 1.385 | 2.728 | 1.343 | 37.2 |
| | 36 | 1.62 | 1.761 | 2.928 | 1.167 | 36.0 |
| | 50 | 2.85 | 0.127 | 0.846 | 0.719 | 14.8 |
| | 68 | 2.73 | 0.027 | 0.466 | 0.438 | 8.8 |

It is evident that even under conditions of unrestricted aeration considerable quantities of a water-soluble metabolic product, presumably mannitol, are formed, and are subsequently destroyed by the mould as the available glucose disappears. The yield of product is not so large, however, as is obtained when the air supply is controlled, and this yield, calculated on the glucose metabolized, decreases *pari passu* with the amount of air supplied.

(b) *Restricted aeration*.—The balance sheet given for Ac. 55 in column 2, Table II, and labelled experiment No. P 18 was prepared from a portion of solution arising as follows :—12 flasks, each containing 250 c.c. of CZAPEK-DOX 5 per cent. glucose medium, were incubated for 77 days and about 300 c.c. of sterile air was passed through each flask each day. At the end of the incubation period the filtered metabolism solution and washings were made up to 4 litres, and of this 250 c.c. were used for preparing the carbon balance sheet, 250 c.c. were used for the mannitol estimations, and the remaining 3,500 c.c. were evaporated *in vacuo* to a syrup which immediately set to a mass of crystals on cooling. This residue was redissolved in boiling water, treated with 100 c.c. of 20 per cent. normal lead acetate solution, and allowed to stand for 3 hours. It was then filtered and the filtrate treated with 100 c.c. of basic lead acetate solution and left overnight. (Treatment of lead precipitates is given later.) In the morning the solution was filtered, excess lead removed from the filtrate with hydrogen sulphide, and the filtrate from the lead sulphide evaporated *in vacuo* to a syrup. The syrup was dissolved in the minimum quantity of boiling 70 per cent. alcohol and, while hot, boiling absolute alcohol was added until a slight permanent turbidity was obtained. The boiling solution was filtered and set aside to crystallise. Mannitol crystallised in beautiful white crystals which were filtered off and dried.

Weight of mannitol, 34·4 gm.

Melting point, 162–163° C.

These crystals gave no reduction of BENEDICT'S solution.

Mother-liquors and washings were evaporated, and a second crop of mannitol weighing 1·95 gm. was obtained. The total yield of mannitol isolated is thus 36·35 gm., representing 77·5 per cent. of the theoretical amount, assuming that all the "carbon unaccounted for" is mannitol, *i.e.*, 47·05 gm. No further mannitol could be isolated from the mother-liquors. It is evident from the above, together with the quantitative figures given in Table II, that mannitol must constitute practically the whole of the "carbon unaccounted for" with Ac. 55. This conclusion is further supported by results given in Part X, p. 201.

The lead precipitates referred to above were too small in amount from this quantity of material for successful investigation, but, as considerable quantities had been accumulated in working up a larger scale experiment with Ac. 55, referred to on p. 170, these were investigated and the treatment of these lead precipitates may with advantage be dealt with here.

The acids were set free from the lead precipitate by treatment with hydrogen sulphide, and a portion of these acids (corresponding to about half of the total, *i.e.*, 30 litres of metabolism solution) was taken for esterification. The aqueous solution of the regenerated acids was evaporated *in vacuo* to a thick syrup, and this was esterified by heating for 5 hours with 250 c.c. of absolute alcohol containing 2·5 per cent. of hydrochloric acid

on three successive occasions—the alcohol being removed by distillation at the end of each period of heating and replaced by fresh alcoholic hydrogen chloride at the start of the next period. The esters were then extracted with ether, the ether solution washed with dilute potassium hydroxide solution and then with water, and dried over anhydrous sodium sulphate. After removal of the ether the esters were fractionated *in vacuo*.

The following fractions were obtained :—

| — | | | | | Boiling Point. | Weight. |
|--------------|-----|-----|-----|-----|-----------------------|-------------|
| | | | | | | Gm. |
| Fraction I | ... | ... | ... | ... | 70°–82° C. at 45 mm. | 3.18 |
| Fraction II | ... | ... | ... | ... | 82°–110° C. at 20 mm. | 1.24 |
| Fraction III | ... | ... | ... | { | 115° C. at 20 mm. | 6.56 |
| | | | | | 111° C. at 13 mm. | |
| Fraction IV | ... | ... | ... | ... | 125° C. at 14 mm. | 1.50 |
| Fraction V | ... | ... | ... | ... | 132° C. at 14 mm. | 1.77 |
| Fraction VI | ... | ... | ... | ... | 173° C. at 17 mm. | A few drops |

The lower boiling fractions of the esters (Fractions I, II and III) were then refractionated at atmospheric pressure, giving

| <i>Boiling Point.</i> | | | | |
|-----------------------|---|--------------|----------|-------------------|
| Fraction | A | 75°–85° C. | 2.58 gm. | } mainly alcohol. |
| „ | B | 85°–98° C. | 1.52 „ | |
| „ | C | 100°–209° C. | 0.58 „ | |
| „ | D | 209°–218° C. | 3.66 „ | |
| (mostly ca. 214°) | | | | |

The fractionation was continued *in vacuo*.

Lower fractions (continued).

| | | | |
|--------------------|----------------------|-----------|----------|
| E | 75°–100° C. (97° C.) | at 15 mm. | 1.30 gm. |
| 4th fraction added | | | |
| F | 90°–115° C. | at 16 mm. | 1.07 gm. |
| 5th fraction added | | | |
| G | 117°–130° C. | at 17 mm. | 1.68 gm. |
| 6th fraction added | | | |
| H | 114°–132° C. | at 14 mm. | 1.33 gm. |
| I | 131°–170° C. | at 15 mm. | 1.25 gm. |

From each of these fractions 1 c.c. was removed and placed in a test tube along with 2 c.c. hydrazine hydrate (50 per cent.) and 2 c.c. absolute alcohol and left overnight. By this treatment the esters of many polybasic acids give rise to the corresponding hydrazides.

D showed crystals (0.14 gm.) which, when filtered and washed, melted at 160°–162° C. The mother-liquor deposited further crystals which melted at 155°–158° C. (0.16 gm.)

E also gave crystals melting at 158°–162° C. which, when recrystallised, melted at 162°–163.5° C.

F gave crystals melting at 155°–158° C.

All these three fractions appeared to give the same hydrazide in different states of purity. It was suspected that this was the dihydrazide of succinic acid, although the melting point was somewhat low. Accordingly 2 gm. of fraction D were hydrolysed with baryta, the acid liberated with sulphuric acid, and extracted with ether. On attempting to sublime the residue from the ether extract a liquid was obtained which distilled, but on cooling crystallised on the sides of the tube. A portion of the crystals was removed and resublimed, and, at about 140° C. a small amount of white sublimate formed which melted at 184° C. (corr.) and was probably succinic acid. A sample of pure succinic acid melted at 185° C. (corr.) M.P. of mixture = 185° C., thus confirming succinic acid. The liquid which distilled was shown to be succinic anhydride after recrystallising a small portion from alcohol; it melted at 117°–119° C. (uncorr.). (M.P. of succinic anhydride = 118°–120° C.). A titration on 0.028 gm. gave an equivalent of 52. Calculated value for succinic anhydride = 50.

Some of the recrystallised hydrazine derivative from Fraction E gave the following combustion figures:—0.1120 gm. hydrazide gave 0.0704 gm. H₂O and 0.1355 gm. CO₂, equivalent to 7.03 per cent. hydrogen and 32.99 per cent. carbon (theoretical for succinic acid dihydrazide is hydrogen 6.90 per cent., carbon 32.87 per cent.).

Fractions G and H were so similar that they were united and treated with hydrazine hydrate. This ester gave a crystalline hydrazide almost immediately on the addition of the hydrazine hydrate. When recrystallised it melted at 180°–181° C. The hydrazide from pure *l*-malic ester melted at 181.5° C. The mixture melted at 181°–182° C., *i.e.*, no lowering of melting point.

The benzylidene compound which was prepared by the method described by RUNDSHAGEN (1926), by dissolving the hydrazide in dilute hydrochloric acid and shaking with benzaldehyde, yielded anomalous results. That prepared from fractions G and H melted at 174° C. after recrystallisation, whereas the derivative from *l*-malic acid melted at 183°–184° C. RUNDSHAGEN gives the melting point as 164°–166° C. Possibly the differences are due to the presence of optical isomers in varying proportions. To confirm malic acid a combustion on the hydrazide was carried out. 0.1027 gm. hydrazide gave 0.0583 gm. H₂O and 0.1116 gm. CO₂.

| | Observed. | Calc. for the dihydrazide of malic acid. |
|-----------|----------------|---|
| H | 6.37 per cent. | 6.22 per cent. |
| C | 29.64 „ | 29.63 „ |

The last fraction, I, did not give a hydrazine derivative except for a very small amount of the dihydrazide of malic acid.

The acids which were thus identified as arising from the growth of Ac. 55 on glucose under conditions of restricted aeration were succinic and malic acids. This constitutes in the carbon balance sheet for Ac. 55 a part of the "carbon as non-volatile acids," but it should be borne in mind that they are relatively so small in amount as to have no appreciable effect on the subsequent recovery of mannitol.

(4) *Aspergillus* species (Ac. 56).

(a) *With varying degrees of aeration.*—The effect of varying degrees of aeration on the growth of Ac. 56 and on the yield of water-soluble metabolic products was investigated in a similar manner to that described for Ac. 55 (see p. 161). The results obtained are given in Table VI, and in this case also all weights are expressed as gm. per 250 c.c. medium. The figures given in the last column represent the amount of mannitol, calculated as gm. carbon per 250 c.c. estimated by the polarimetric method given in Part X.

TABLE VI.—Effect of aeration on yield of Metabolic Products of Ac. 56.

| Degree of aeration. | Incubation period in days. | Weight of mycelium. | Carbon in glucose. | Total carbon in solution. | Difference, i.e., carbon as metabolic products in solution. | Percentage yield of metabolic products calculated on glucose consumed. | Carbon as mannitol estimated polarimetrically. |
|---------------------------|----------------------------|---------------------|--------------------|---------------------------|---|--|--|
| | | gm. | gm. | gm. | gm. | | gm. |
| 100 c.c.... | 40 | 0.34 | 3.812 | 4.485 | 0.673 | 56.6 | 0.231 |
| 100 c.c.... | 69 | 0.47 | 3.258 | 4.138 | 0.880 | 50.5 | — |
| 200 c.c.... | 40 | 0.49 | 3.018 | 4.165 | 1.147 | 57.9 | — |
| 200 c.c.... | 69 | 0.74 | 2.052 | 3.607 | 1.555 | 52.8 | 0.647 |
| 300 c.c.... | 40 | 0.59 | 3.039 | 3.948 | 0.909 | 46.4 | — |
| 300 c.c.... | 69 | 0.94 | 1.393 | 3.212 | 1.819 | 50.4 | 0.725 |
| 400 c.c.... | 40 | 0.67 | 2.572 | 3.851 | 1.279 | 52.7 | 0.493 |
| 400 c.c.... | 68 | 0.99 | 1.155 | 3.036 | 1.881 | 48.9 | — |
| 600 c.c.... | 40 | 0.78 | 2.373 | 3.692 | 1.318 | 50.2 | — |
| 600 c.c.... | 68 | 1.26 | 0.596 | 2.643 | 2.047 | 46.5 | 0.631 |
| 800 c.c.... | 40 | 0.96 | 2.119 | 3.436 | 1.317 | 45.7 | — |
| 800 c.c.... | 68 | 1.59 | 0.139 | 2.086 | 1.947 | 40.1 | 0.558 |
| Unrestricted aeration ... | 32 | 2.31 | 0.257 | 1.981 | 1.724 | 36.3 | — |

The results given in Table VI indicate that, as was the case with Ac. 55, the yield of water-soluble metabolic products obtained is very greatly influenced by the degree of aeration. The figures given in the last column show that, unlike Ac. 55, metabolic products other than mannitol are formed by Ac. 56, since mannitol only forms 30-40 per cent. of the total metabolic products of Ac. 56 over a wide range of air supply.

(b) *With restricted aeration.*—Nine litres of 5 per cent. CZAPEK-DOX glucose medium were made up and 250 c.c. of this distributed in each of 36 flasks. 300 c.c. of sterile air were passed through each flask daily, and at the end of the incubation period the metabolism solutions were mixed, filtered, and filtrate and mycelium washings neutralised with sodium hydroxide to $pH\ 7.0$, and made up to 12 litres. Of this, 250 c.c. were used for the preparation of the balance sheet given in Table II, column 2, and 250 c.c. for the mannitol estimations given in the same column. The remaining 11.5 litres were treated with normal and basic lead acetate, filtered, the lead removed from the filtrate by hydrogen sulphide, and the lead-free filtrate evaporated *in vacuo* to a syrup which crystallised on standing. The crystalline mass was then thoroughly extracted with 400 c.c. of absolute alcohol. The mixture was filtered and the crystals washed and dried. They consisted of fairly pure mannitol, and weighed 67 gm., corresponding to 43.1 per cent. of the total "carbon unaccounted for," and 67.9 per cent. of the mannitol estimated to be present by polarimeter. It is evident that with Ac. 56 a considerable proportion of the "carbon unaccounted for" is some product other than mannitol, a conclusion which is supported by the results given in Table VI.

The syrupy filtrate from the mannitol crystals was diluted to rather more than a litre with absolute alcohol, when a certain amount of sticky material was precipitated. The clear alcoholic solution was poured off, evaporated *in vacuo* and made up again to about a litre with fresh absolute alcohol. A further precipitate was produced, and the clear alcoholic solution was again separated and to it were added 2 litres of ether. A third precipitate was thus obtained and the ether-alcohol solution was poured off and evaporated *in vacuo*. The residue from this was further purified by subjecting it to a second precipitation, by redissolving in absolute alcohol and reprecipitating with two volumes of dry ether. A thick sticky residue was deposited, and the clear ether-alcohol solution was poured off and evaporated, giving rise to a clear syrup having a weight of 10 gm. Treatment of this dry syrup will be given later.

The three sticky precipitates contained considerable amounts of glucose. They were collected together and fermented for three days with a pure culture of yeast. The fermented solution was filtered, cleared with lead acetate, and the lead-free filtrate evaporated to a thick syrup. This was then subjected to alcohol-ether treatment as described above, giving rise to a second clear syrup, the weight of which was 3.5 gm.

The two syrups described above, weighing respectively 10 gm. and 3.5 gm., were shown to consist almost entirely of glycerol by the following means.

- (a) The mean molecular weight of a portion of the syrups was determined by estimating the depression of the freezing point of water. They gave a mean value of about 80 (molecular weight of pure glycerol = 92).
- (b) A portion of each syrup was benzoylated by shaking an aqueous solution of it with benzoyl chloride and sodium hydroxide solution. An almost quantitative yield of glycerol tribenzoate was obtained having the following

characteristics:—Melting point, 71.5° – 72° C. A synthetic sample of pure glycerol tribenzoate gave a melting point of 71° – 72° C., and a mixture of the benzoate isolated from the syrup and of synthetic glycerol tribenzoate had a melting point of 70° – 71° C.

Combustions on the benzoate isolated from the syrup gave the following results.

| | Combustion (1) | Combustion (2) | Theoretical for glycerol tribenzoate. |
|-----------|----------------|----------------|--|
| C | 71.6 per cent. | 71.2 per cent. | 71.2 per cent. |
| H | 5.06 ,, | 5.12 ,, | 4.99 ,, |

Further proof of the presence of glycerol was obtained as follows:—

5.6 gm. of this syrup (first crop) were used for a phenyl isocyanate reaction. The only products which could be identified after repeated recrystallisation of the mixture were glycerol triphenylurethane and diphenyl urea.

In a further attempt to gain some information as to whether any product other than glycerol was present in the syrup, a portion of it was subjected to the action of periodic acid by the method of MALAPRADE (1928).

Two figures were obtained for the amount of glycerol present, one from the amount of periodic acid reduced to iodic acid and the other from the titratable acidity produced by the reaction. This method, whilst admittedly not very accurate, is at any rate more specific than the rest of the glycerol methods and enables a distinction to be made between glycerol and ethylene glycol, since the latter produces no acidity.

Glycerol by reduction of HIO_4 = 92.7 per cent.

Glycerol by acidity produced = 89.7 ,,

Making due allowance for the error of the method, it may be said that the agreement is good, and that the syrup remaining after removal of mannitol consists of glycerol. Any other substances, except water, are present only in negligible amounts.

Hence, unlike Ac. 55, for which mould the “carbon unaccounted for” is almost entirely mannitol, with Ac. 56 rather more than half of the “carbon unaccounted for” is mannitol and a considerable proportion of the remainder, if not all of it, is glycerol.

The acids precipitated as lead salts, as described on p. 163, were worked up in a similar manner to that adopted for the lead precipitate of Ac. 55 described on p. 164. The only hydrazide isolated from the acids from Ac. 56 was one having a melting point of 178° – 179° C. The hydrazide of pure *l*-malic acid melts at 181.5° C. and a mixture of the hydrazide isolated and of synthetic *l*-malic acid hydrazide melted at 179° C. The benzyldene derivative prepared from the above hydrazide melted at 166° – 168° C. This agrees substantially with the melting point given by RUNDSHAGEN for the *l*-malic acid derivative (164° – 166° C.) though it disagrees with the melting points observed here and given on p. 165. The explanation is probably that offered on p. 165.

The only acid identified as arising from the growth of *Ac. 56* on glucose under conditions of restricted aeration was thus *l*-malic acid.

Production of Glycogen by Aspergillus species Ac. 56.

In an experiment with *Aspergillus* species *Ac. 56*, which was carried out in the combined sterilizer incubator described in Part VII, the production of a polysaccharide was observed, which on investigation proved to be glycogen. The experimental details follow :—

One tray containing five litres of CZAPEK-Dox 5 per cent. glucose medium, with 2 gm. per litre of ammonium nitrate in place of the usual 2 gm. of sodium nitrate, was used for this experiment. The actual nitrogen present was thus about twice the standard amount. After sterilization the medium was sown with *Ac. 56* and aerated at the rate of 0.25 cu. ft. per day at room temperature. The incubation period lasted 47 days. The contents of the tray were shown to be free from contamination, and the filtered metabolism solution then showed a strong opalescence and gave a characteristic red-brown reaction with iodine. It was evaporated *in vacuo* and an equal volume of alcohol was added. An amorphous white powder was precipitated which was very difficult to filter. It was washed by centrifuging with alcohol and ether and dried. Yield of crude material containing 9.5 per cent. of ash = 7 gm.

The substance was purified as follows : It was dissolved in hot water, filtered from the insoluble portion, acidified with 5 c.c. concentrated HCl and precipitated by the addition of 1.5 volumes of 96 per cent. alcohol. It was filtered off, drained, and reprecipitated twice from *neutral* solution with an equal volume of alcohol, washed with alcohol and ether and dried.

The final product is a pure white amorphous powder containing no ash and neutral in reaction. Its aqueous solution is slightly opalescent in the cold and gives a characteristic brownish-red colour with iodine.

0.2280 gm. was dissolved in hot water, made up to 50 c.c., and polarised in a 20-cm. tube. The readings obtained were $+1.753^\circ$ with the mercury yellow light corresponding to $[\alpha]_{\text{Hg. yellow}}^{20} = +192.2^\circ$ and $+1.932^\circ$ with the mercury green light corresponding to $[\alpha]_{\text{Hg. green}}^{20} = +211.9^\circ$.

0.4028 gm. was hydrolysed by heating with 10 c.c. of N/1 H_2SO_4 for four hours on a boiling water bath. The hydrolysis mixture was neutralised, made up to 50 c.c. and filtered. The glucose content as estimated by the polarimeter was 0.836 per cent. and by the WOOD-OST method 0.826 per cent.

As the sugar formed on hydrolysis was definitely proved to be glucose, it is evident from these figures that the material is a polyglucose, and from its optical rotation and its colour reaction with iodine it is either identical with, or closely allied to, glycogen.

Large Scale Experiments on the Production of Mannitol by Aspergillus species Ac. 55.

Experiments on a 60 litre scale were carried out in the combined sterilizer-incubator described in detail in Part VII of this series.

Five litres of CZAPEK-DOX 5 per cent. glucose solution ($pH=7.4$) were placed in each of the twelve trays. Each tray was also fitted with a support for the mycelium, consisting of a grid of aluminium wire-netting of 1 inch mesh, the edges of which were turned over so that the grid was supported just below the surface of the liquid. This grid was found to be necessary in order to prevent the mycelium sinking into the liquid during the manipulations described later. After sterilizing and sowing the trays with *Ac. 55*, the whole apparatus was incubated at room temperature for a period of 59 days and during the whole incubation period sterile air at the rate of 0.25 cubic feet per tray was passed daily.

At the end of the incubation period the metabolism solution was removed through the inoculation openings by means of sterile syphons, care being taken to disturb the mycelium as little as possible.

The metabolism solution was treated with basic and neutral lead acetates in the usual way, the washed lead precipitates being examined by the method already described on page 163. The filtrate from the lead precipitates was freed from lead, evaporated, and a total yield of 608 gms. of mannitol was isolated corresponding to about 24 per cent. of the glucose fermented.

An experiment was now carried out to determine the effect of replacing the withdrawn metabolism solution by fresh sterile CZAPEK-DOX 5 per cent. glucose solution. The experimental details follow. After removal of the exhausted metabolism solution as described above, each tray was washed out with 4 litres of sterile distilled water, introduced beneath the mycelium, and removed by means of sterile syphons. Five litres of fresh sterile CZAPEK-DOX 5 per cent. glucose solution were now introduced into each tray beneath the mycelium. It was possible to carry out these manipulations without submerging the mycelium because of the support given to the mycelial felt by the aluminium grids described.

Incubation at room temperature and aeration as previously described were carried out for a period of 25 days. By this time almost all the glucose had disappeared in most of the trays and yields of mannitol varying between 39.9 per cent. and 50.2 per cent., calculated on the glucose metabolized, were obtained. As was inevitable, contaminations were found in a small proportion of the trays, but in the uncontaminated trays an average yield of 46.3 per cent. of mannitol was obtained.

It is thus evident that by the use of a fresh medium with a mycelial felt already established, not only is the incubation period necessary for the fermentation of a given quantity of glucose considerably curtailed, but the yield of mannitol obtained is considerably increased. It is also significant that, even under these optimum conditions, the yields of mannitol obtained definitely tend to a maximum of 50 per cent. of the

glucose fermented corresponding to the production of one molecule of mannitol from two molecules of glucose. This lends support to the view expressed on p. 9 of the introductory paper (Part I) of this series that the initial step in the metabolism of glucose by certain species of fungi is a CANNIZZARO reaction involving the production from two molecules of glucose of one molecule of mannitol and one molecule of gluconic acid.

Summary.

The formation of mannitol from glucose by three unnamed white species of *Aspergillus*, one strain of *A. elegans*, and five different strains of *A. nidulans*, has been investigated.

The fundamental effect of variations in the air supply on the yield of mannitol obtained is described.

It has been found that a well-developed mycelium of the white species Ac. 55 may be used to ferment to mannitol a fresh quantity of glucose solution, supplied in place of the exhausted medium. By this means, yields of mannitol approaching 50 per cent. of the glucose fermented were obtained.

Other metabolic products found in addition to mannitol were :

- (a) Small quantities of succinic acid and malic acid produced by the white species Ac. 55.
- (b) A considerable quantity of glycerol, a complex carbohydrate closely resembling, if not identical with, glycogen and small quantities of *l*-malic acid produced by the white species Ac. 56.

*Studies in the Biochemistry of Micro-organisms.*PART X.—*The Estimation of Mannitol in Fermentation Solutions.*

By HAROLD RAISTRICK and WILLIAM YOUNG.

This paper deals with the problem of estimating mannitol in the presence of glucose and of products arising during the fermentation of this sugar.

Work was started by preparing synthetic mixtures of pure mannitol and pure glucose, and, a satisfactory method of estimating each of these in presence of the other having been found, the effect of the presence of other fermentation products, *e.g.*, glycerol, on the accuracy of the method was tested. A successful solution of the problem was found in the use of the polarimeter. Details of the polarimetric work carried out on pure mannitol and glucose are given on pp. 173–185. Chemical methods which were also tried on these substances were :—

- (1) Estimation of mannitol as the tribenzoyl acetal, or as the *p*-nitrobenzoyl derivative (see p. 185).
- (2) Estimation of mannitol by acetylation (see pp. 185–187).

The use of the polarimetric and acetylation methods in conjunction with each other on synthetic mixtures of mannitol and glucose was next tried and is described on pp. 187–198, and then the application of these methods to actual fermentation solutions was investigated, the results being given on pp. 200–207. Finally, a quicker but somewhat less accurate method for the estimation of mannitol in fermentation solutions, which obviates the time-consuming step of removing glucose by fermentation, was devised for routine use and is discussed on pp. 198–200.

SECTION A.—POLARIMETRIC WORK ON PURE MANNITOL AND PURE GLUCOSE.

Mannitol in solution in pure water has no measurable rotation. The addition of borax, however, renders the solution optically active. Preliminary tests were therefore carried out to find the maximum concentration of borax which would give no trouble by crystallising out at room temperature, and it was found that with a solution containing 6 gm. of borax per 100 c.c. no trouble was experienced. As this concentration of borax was found to give satisfactorily large rotations, it was adopted throughout the

work. The following figures, obtained with mercury yellow light, give an idea of the effect of varying the concentration of borax and also of the magnitude of the rotations :—

| Gm. Mannitol per 100 c.c. | °Rotation, with Concentration of Borax Decahydrate per 100 c.c. | | |
|---------------------------|---|----------|----------------------------------|
| | 7.50 gm. | 6.00 gm. | Differences. |
| 0.700 | 1.020 | 1.005 | $D_1 \quad D_2$ 0.015 > 0.047 |
| 1.700 | 2.318 | 2.256 | 0.062 > 0.110 |
| 2.700 | 3.422 | 3.250 | 0.172 |

Each figure is the average of at least six readings and, as the error in taking the end point is less than $\pm 0.01^\circ$, it will be seen that the differences between the two sets of rotations is quite distinct. It will be noticed also that there is a distinct difference between the second differences, D_2 , for equal increments of mannitol.

Throughout this work pure borax decahydrate ($\text{Na}_2\text{B}_4\text{O}_7 \cdot 10 \text{H}_2\text{O}$) was used and was dried to constant weight over sulphuric acid of not more than 70 per cent. strength.

(a) *Construction of a Table connecting Concentration of Mannitol in 6 per cent. Borax Solution with its Rotation.*

Tests were carried out to determine the rotation in 6 per cent. borax solution of concentrations of mannitol from 0.1 to 5.0 per cent. The mannitol used was material supplied by British Drug Houses, further purified by one recrystallisation from water, one from 80 per cent. alcohol and two more from water. Throughout this work readings were taken in a Hilger Standard Polarimeter of 400 mm. length and fitted with a direct vision spectroscopic attachment for working with the light from the mercury green and yellow lines. As a source of illumination for the polarimeter a quartz atmospheric mercury-vapour lamp supplied by Messrs. Kelvin, Bottomley and Baird was used. With these instruments an accuracy of 0.01° is easily obtained. The results are given in the tables to three places of decimals, which figures were arrived at by taking the average of six to ten consecutive readings. The working tables were constructed correspondingly.

Rotations were taken with both the yellow and the green light. The former light is very intense and is useful for work with coloured solutions; the latter light is that usually reported in the literature. The figures obtained are shown in Table I. It is also shown in this table that the ratio between the two sets of rotations may be accepted as constant, the value being 1.1193. Curves were drawn with rotations plotted against concentration of mannitol and the yellow light was found to have given rather steadier results than the green light. The yellow light curve was therefore smoothed and from the smoothed curve Table II was drawn up

TABLE I.—Rotation of Mannitol in 6.00 per cent. Borax Solution, with Mercury Yellow and Green Lights. A 4 dm. tube was used.

| Gm. Mannitol in 100 c.c. | °Rotation. | | | | | |
|-----------------------------|---------------|-----------|---------------------|--------------|--------------------------------------|-------------|
| | Yellow Light. | | | Green Light. | | |
| | Filtered. | Resolved. | Mean of A and B. | Found. | Calc. from C. by factor 1.1193 | Difference. |
| | A. | B. | C. | D. | E. | D — E. |
| 0.1005 | 0.152 | 0.148 | 0.150 | 0.158 | 0.168 | — 0.010 |
| 0.3000 | 0.447 | 0.445 | 0.446 | 0.485 | 0.499 | — 0.014 |
| 0.4999 | 0.732 | 0.730 | 0.731 | 0.807 | 0.818 | — 0.011 |
| 0.7004 | 1.008 | 1.008 | 1.008 | 1.133 | 1.128 | + 0.005 |
| 0.9005 | 1.260 | 1.268 | 1.264 | 1.397 | 1.415 | — 0.018 |
| 1.1008 | 1.530 | 1.527 | 1.529 | 1.668 | 1.711 | — 0.043 |
| 1.3004 | 1.783 | 1.785 | 1.784 | 1.965 | 1.996 | — 0.030 |
| 1.4999 | 2.012 | 2.020 | 2.016 | 2.250 | 2.256 | — 0.006 |
| 1.6826 | 2.235 | 2.238 | 2.236 | 2.482 | 2.502 | — 0.020 |
| 1.9065 | 2.465 | 2.478 | 2.472 | 2.767 | 2.767 | 0.000 |
| 2.1003 | 2.685 | 2.685 | 2.685 | 3.005 | 3.004 | + 0.001 |
| 2.2940 | 2.873 | 2.875 | 2.874 | 3.220 | 3.217 | + 0.003 |
| 2.5011 | 3.075 | 3.078 | 3.077 | 3.448 | 3.443 | + 0.005 |
| 2.6965 | 3.223 | 3.243 | 3.233 | 3.635 | 3.618 | + 0.017 |
| 2.9039 | 3.412 | 3.425 | 3.419 | 3.835 | 3.826 | + 0.009 |
| 3.1001 | 3.600 | 3.607 | 3.603 | 4.052 | 4.033 | + 0.019 |
| 3.2969 | 3.747 | 3.755 | 3.751 | 4.192 | 4.198 | — 0.006 |
| 3.5041 | 3.877 | 3.895 | 3.886 | 4.366 | 4.349 | + 0.017 |
| 3.7010 | 4.025 | 4.022 | 4.024 | 4.512 | 4.502 | + 0.010 |
| 3.8994 | 4.150 | 4.158 | 4.154 | 4.658 | 4.648 | + 0.010 |
| 4.1002 | 4.285 | 4.285 | 4.285 | 4.808 | 4.795 | + 0.013 |
| 4.3011 | 4.383 | 4.385 | 4.384 | 4.925 | 4.907 | + 0.018 |
| 4.5006 | 4.497 | 4.495 | 4.496 | 5.040 | 5.031 | + 0.009 |
| 4.7010 | 4.577 | 4.580 | 4.577 | 5.142 | 5.124 | + 0.018 |
| 4.8966 | 4.673 | 4.677 | 4.675 | 5.248 | 5.232 | + 0.016 |
| 5.0009 | 4.710 | 4.712 | 4.711 | 5.273 | 5.272 | + 0.001 |

Total 75.470 84.471

$$\text{Ratio } \frac{84.471}{75.470} = 1.1193.$$

TABLE II.—Connecting °Rotation with gm. Mannitol in 100 c.c. Solution.

| <i>Conditions :—</i> | | | | | |
|--|---------------|--------------------|--------------|---------------|--------------------|
| 1. The mannitol solution contains 6.00 gm. $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$ per 100 c.c. | | | | | |
| 2. A 4 dm. tube is used. | | | | | |
| Yellow Light. | | | Green Light. | | |
| °Rotation. | Gm. Mannitol. | Δ for 0.1°. | °Rotation. | Gm. Mannitol. | Δ for 0.1°. |
| 0.1 | 0.064 | 0.065 | 0.1 | 0.057 | 0.058 |
| 0.2 | 0.129 | 0.067 | 0.2 | 0.115 | 0.060 |
| 0.3 | 0.196 | 0.068 | 0.3 | 0.175 | 0.060 |
| 0.4 | 0.264 | 0.069 | 0.4 | 0.235 | 0.061 |
| 0.5 | 0.333 | 0.070 | 0.5 | 0.286 | 0.062 |
| 0.6 | 0.403 | 0.072 | 0.6 | 0.358 | 0.064 |
| 0.7 | 0.475 | 0.073 | 0.7 | 0.422 | 0.064 |
| 0.8 | 0.548 | 0.074 | 0.8 | 0.486 | 0.065 |
| 0.9 | 0.622 | 0.074 | 0.9 | 0.551 | 0.066 |
| 1.0 | 0.696 | 0.075 | 1.0 | 0.617 | 0.066 |
| 1.1 | 0.771 | 0.075 | 1.1 | 0.683 | 0.067 |
| 1.2 | 0.846 | 0.076 | 1.2 | 0.750 | 0.067 |
| 1.3 | 0.922 | 0.077 | 1.3 | 0.817 | 0.068 |
| 1.4 | 0.999 | 0.078 | 1.4 | 0.885 | 0.068 |
| 1.5 | 1.077 | 0.079 | 1.5 | 0.953 | 0.069 |
| 1.6 | 1.156 | 0.080 | 1.6 | 1.022 | 0.070 |
| 1.7 | 1.236 | 0.081 | 1.7 | 1.092 | 0.071 |
| 1.8 | 1.317 | 0.082 | 1.8 | 1.163 | 0.071 |
| 1.9 | 1.399 | 0.082 | 1.9 | 1.234 | 0.072 |
| 2.0 | 1.481 | 0.084 | 2.0 | 1.306 | 0.073 |
| 2.1 | 1.565 | 0.086 | 2.1 | 1.379 | 0.074 |
| 2.2 | 1.651 | 0.088 | 2.2 | 1.453 | 0.074 |
| 2.3 | 1.739 | 0.090 | 2.3 | 1.527 | 0.076 |
| 2.4 | 1.829 | 0.092 | 2.4 | 1.603 | 0.077 |
| 2.5 | 1.921 | 0.095 | 2.5 | 1.680 | 0.079 |
| 2.6 | 2.016 | 0.098 | 2.6 | 1.759 | 0.081 |
| 2.7 | 2.114 | 0.102 | 2.7 | 1.840 | 0.082 |
| 2.8 | 2.216 | 0.104 | 2.8 | 1.922 | 0.085 |
| 2.9 | 2.320 | 0.106 | 2.9 | 2.007 | 0.088 |
| 3.0 | 2.426 | 0.108 | 3.0 | 2.095 | 0.090 |
| 3.1 | 2.534 | 0.110 | 3.1 | 2.185 | 0.092 |
| 3.2 | 2.644 | 0.112 | 3.2 | 2.277 | 0.094 |
| 3.3 | 2.756 | 0.115 | 3.3 | 2.371 | 0.096 |
| 3.4 | 2.871 | 0.119 | 3.4 | 2.467 | 0.097 |
| 3.5 | 2.990 | 0.124 | 3.5 | 2.564 | 0.098 |
| 3.6 | 3.114 | 0.128 | 3.6 | 2.662 | 0.100 |
| 3.7 | 3.242 | 0.132 | 3.7 | 2.762 | 0.103 |
| 3.8 | 3.374 | 0.137 | 3.8 | 2.865 | 0.106 |
| 3.9 | 3.511 | 0.144 | 3.9 | 2.971 | 0.110 |
| 4.0 | 3.655 | — | 4.0 | 3.081 | 0.114 |
| | | | 4.1 | 3.195 | 0.116 |
| | | | 4.2 | 3.311 | 0.120 |
| | | | 4.3 | 3.431 | 0.125 |
| | | | 4.4 | 3.556 | 0.130 |
| | | | 4.5 | 3.686 | — |

giving gm. mannitol per 100 c.c. for every 0.1° of rotation. The corresponding figures for the green light were deduced by means of the factor 1.1193 given in Table I, and are also given in Table II.

In Table I, the yellow light is described as (1) filtered, and (2) resolved. When the polarimeter is not fitted with a resolving prism, a bichromate filter is used and thus filtered yellow light is obtained. When the direct vision spectroscope is used, the bichromate filter is removed and both yellow and green lights are obtainable, separated by the action of the prism. It will be seen that the two yellow lights give the same rotation.

In order to determine whether the factor 1.1193 shown in Table I is independent of the borax concentration, a test was carried out in which the borax concentration was doubled. This was rendered possible because of the increased solubility of borax in aqueous mannitol solutions compared with water only. The results were as follows :

| Per 100 c.c. | | °Rotation. | | |
|---------------|------------|---------------|-----------|--------------|
| Gm. Mannitol. | Gm. Borax. | Yellow Light. | | Green Light. |
| | | Filtered. | Resolved. | |
| 10.001 | 12.00 | 9.478 | 9.472 | 10.627 |
| | | Mean = 9.475 | | |

$$\text{Ratio } \frac{10.627}{9.475} = 1.1215 \quad (\text{compare } 1.1193 \text{ from Table I}).$$

The difference is 0.2 per cent., so that the ratio seems to be independent of the concentration of borax.

(b) The Effect of Glycerol on the Rotation of Mannitol in Borax Solution.

During the work on chemical methods of estimating mannitol, indications were found that glycerol was present at a stage where the polarimeter would be used. The effect of glycerol on the rotation of mannitol in borax solution was therefore tested. The results are given in Table III.

It is evident that glycerol reduces the rotation of mannitol. In practice, however, the glycerol concentration is in general too small compared with the mannitol concentration to cause any appreciable error in the polarimeter test. As shown later, efforts were made to remove this source of error entirely.

(c) The Effect of Glucose on the Rotation of Mannitol in 6 per cent. Borax Solution.

An attempt to estimate mannitol in glucose-mannitol mixtures, without removing the glucose, led to a study of the effect of glucose on the rotation of mannitol in 6 per cent. borax solution. The first part of the work dealt with the effect of borax on the rotation of glucose itself.

The Effect of Borax on the Rotation of Glucose.

The ratio of the rotations of glucose in pure water solution with the yellow and green lights was obtained first of all, the results being :—

| Gm. Glucose per 100 c.c. | | °Rotation. | | | Ratio. |
|-----------------------------------|--------|-----------------------|-----------|----------------|--------|
| | | Yellow Light. | | Green light | |
| | | Filtered. | Resolved. | | |
| (1) | 3.9968 | 8.700 Mean = 8.702 | 8.704 | 9.875 | 1.1348 |
| (2) | 1.9990 | 4.340 Mean = 4.345 | 4.350 | 4.935 | 1.1358 |
| Mean Ratio = 1.1353 | | | | | |
| Compare Mannitol result (Table I) | | | | | 1.1193 |

The effect of borax on the rotation of glucose was now investigated. 26.017 gm. of glucose, purified by recrystallisation from aqueous alcohol, were dissolved in water and the solution made up to 250 c.c. The solution was allowed to stand until its rotation was constant. The effect of the borax on the rotation of glucose was studied by taking equal quantities of the above solution and making up to 100 c.c., 6 per cent. of borax being added in one set and omitted from the parallel set. The results are given in Table IV.

The calculated and experimental results agree quite well for both wavelengths used, when borax is absent. When borax is present, the reduction amounts in one case to 3.5°, while an almost zero reading is obtained for 1 per cent. of glucose which would, in the absence of borax, give a reading of 2.3°.

In Test 6, two solutions were prepared, one in which solution of the borax in the glucose solution was effected on a boiling water bath, the other at 35° C. There is little difference in the results.

In view of the results obtained, a second solution of glucose was prepared. The accuracy of the solution was checked polarimetrically without borax present, after which only tests with borax were carried out. The results are given in Table V.

TABLE IV.—Effect of Borax on the Rotation of Glucose.

| Test number : | 1 | 2 | 3 | 4 | 5 | 6 |
|---|--------|--------|--------|--------|--------|--------------|
| Gm. glucose per 100 c.c. : | 5.203 | 5.203 | 2.602 | 2.602 | 1.0407 | 1.0407 |
| Gm. $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$ per 100 c.c. : | Nil | 6.00 | Nil | 6.00 | Nil | 6.00 |
| $^{\circ}$ Rotation. | | | | | | |
| Hg. light— | | | | | | I. II. |
| Yellow— | | | | | | |
| Found | 11.305 | 8.262 | 5.655 | 2.010 | 2.268 | 0.040 0.031 |
| Calculated | 11.369 | 11.369 | 5.685 | 5.685 | 2.273 | 2.273 2.273 |
| Difference... .. | —0.064 | —3.103 | —0.030 | —3.675 | —0.005 | —2.233—2.242 |
| Green— | | | | | | |
| Found | 12.922 | — | 6.440 | — | 2.612 | — |
| Calculated | 12.903 | — | 6.452 | — | 2.581 | — |
| Difference | +0.019 | — | —0.012 | — | +0.031 | — |

Calculated values for Hg. green light were obtained from formula :— $62lw = 100\alpha$ (1)

Calculated values for Hg. yellow light were obtained from formula :— $62lw = 100\alpha \times 1.1353$ (2)

In formula (1) the factor 62 was obtained from S. W. COLE's *Pract. Physiol. Chem.*, 5th Ed. (1919), p. 417.

In formula (2) the factor 1.1353 was obtained from the tests on p. 179 of this paper.

A 4 dm. tube was used in the tests so that in the formulæ, $l = 4$.

TABLE V.—A Repetition of Table IV, showing further the Effect of 6 per cent. of Borax on the Rotation of Glucose.

| Test number : | 1 | 2 | 3 | 4 | 5 |
|---|---------|---------|---------|---------|---------|
| Gm. glucose per 100 c.c. : | 5.209 | 5.209 | 2.605 | 1.042 | 0.521 |
| Gm. $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$ per 100 c.c. : | Nil | 6.00 | 6.00 | 6.00 | 6.00 |
| $^{\circ}$ Rotation. | | | | | |
| Hg. light— | | | | | |
| Yellow— | | | | | |
| Found | 11.318 | 8.260 | 2.028 | + 0.025 | — 0.015 |
| Calculated | 11.380 | 11.380 | 5.690 | 2.276 | 1.138 |
| Difference | — 0.062 | — 3.120 | — 3.662 | — 2.251 | — 1.153 |
| Green— | | | | | |
| Found | 12.910 | 9.272 | 2.235 | + 0.028 | — 0.122 |
| Calculated | 12.918 | 12.918 | 6.459 | 2.584 | 1.292 |
| Difference | — 0.008 | — 3.646 | — 4.234 | — 2.556 | — 1.414 |

These results agree with those of the previous table, and leave no doubt that the rotation of glucose is lowered by the presence of borax, a zero result being obtained somewhere about 1 per cent. of glucose and the rotation becoming negative with lower concentrations. Some of the readings were repeated after 24 hours, but no change was found. A further series of tests was therefore carried out in order to construct a curve, and the portion between 0.1 and 1.0 per cent. of glucose was specially studied. The results of the tests are given in Table VI.

TABLE VI.—Rotation of Glucose in 6 per cent. Borax Solution for Concentrations of Glucose between 0.1 per cent. and 5.0 per cent.

| Percentage Glucose. | °Rotation with Mercury Light. | |
|---------------------|-------------------------------|---------|
| | Yellow. | Green. |
| 0.1 | — 0.033 | — 0.037 |
| 0.2 | — 0.059 | — 0.075 |
| 0.3 | — 0.085 | — 0.093 |
| 0.4 | — 0.112 | — 0.125 |
| 0.5 | — 0.115 | — 0.124 |
| 0.6 | — 0.103 | — 0.113 |
| 0.7 | — 0.084 | — 0.103 |
| 0.8 | — 0.041 | — 0.044 |
| 0.9 | + 0.005 | + 0.003 |
| 1.0 | + 0.030 | + 0.029 |
| 1.5 | + 0.315 | + 0.352 |
| 2.0 | + 0.905 | + 1.000 |
| 2.5 | + 1.655 | + 1.835 |
| 3.0 | + 2.698 | + 2.967 |
| 3.5 | + 3.743 | + 4.132 |
| 4.0 | + 4.993 | + 5.575 |
| 5.0 | + 7.493 | + 8.417 |

As a further check on the above negative rotations, two tests were carried out with the addition compound of glucose and sodium chloride, $2C_6H_{12}O_6 \cdot NaCl \cdot H_2O$, which is easily obtained in a pure state. The results were as follows:—

| Test No. | Gm. Glucose Sod. Chloride per 100 c.c. | Gm. Borax per 100 c.c. | °Rotation Hg. Yellow Light. | |
|----------|--|---------------------------|--------------------------------|---------|
| | | | Found. | Calc. |
| 1 | 0.658 | Nil | + 1.187 | + 1.183 |
| 2 | 0.627 | 6.00 | — 0.110 | — |

A second sample of glucose six times recrystallised from aqueous alcohol showed the following results :—

| Glucose gm. per 100 c.c. | Borax gm. per 100 c.c. | °Rotation. | |
|-----------------------------|---------------------------|------------|--------------------|
| | | Found. | Calc. |
| 0.500 | Nil | 1.085 | 1.092 Yellow Light |
| | | 1.235 | 1.240 Green Light |
| 0.500 | 6.00 | — 0.115 | Yellow Light |
| | | — 0.125 | Green Light |
| 0.900 | 6.00 | — 0.005 | Yellow Light |
| | | — 0.005 | Green Light |

These results agree very well with those previously found, and it may be accepted that borax has the following effects on the rotation of glucose in aqueous solution :—

- (1) Borax reduces the rotation of glucose at all concentrations studied.
- (2) At a concentration of 0.9 per cent. glucose and 6.00 per cent. borax, the solution is optically inactive.
- (3) At concentrations below 0.9 per cent. glucose with 6.00 per cent. borax, the solution actually becomes laevorotatory, reaching a maximum laevorotation at about 0.5 per cent. glucose.

We have no definite explanation to offer for these observations, though it is possible that the action of borax on glucose in aqueous solution may produce a LOBRY DE BRUYN transformation, although this explanation seems inadequate since the effect of the borax is practically instantaneous.

These results indicated a possible polarimetric method for the estimation of mannitol in aqueous solutions containing glucose, without the necessity of prior removal of glucose. It will be seen that by adding borax to a concentration of 6 per cent. to the solution under investigation after adjusting the glucose concentration to 0.9 per cent. either by dilution if the concentration of the glucose is greater than 0.9 per cent. or by the addition of glucose if the test solution contains less than 0.9 per cent. of glucose, the optical effect of any glucose present will be reduced to a minimum. It is thus possible to remove errors due to the relatively low optical rotation of mannitol, compared with that of glucose. In those cases where the ratio of mannitol to glucose present is relatively large, it was felt that greater accuracy would be attained by reducing the glucose to 0.5 per cent. instead of 0.9 per cent. For this reason, therefore, the following two series of estimations (Tables VII and IX) were carried out to determine :—

1. The optical rotation of aqueous solutions of mannitol in presence of 6 per cent borax and 0.9 per cent. glucose.

2. The optical rotation of aqueous solutions of mannitol in presence of 6 per cent. borax and 0.5 per cent. glucose.

The results obtained and the working tables deduced from them are given below.

Construction of Tables connecting Rotation with Concentration of Mannitol in 6 per cent. Borax and 0.9 per cent. Glucose Solution.

The experimental results found in this connection are given in Table VII. The glucose was added in the form of a 3.6 per cent. solution, of which 25 c.c. were used in each test in Table VII. The 3.6 per cent. solution was tested polarimetrically, and found to show in a 4 dm. tube a rotation of 8.925° with the mercury green light corresponding to 3.598 gm. of glucose per 100 c.c.

TABLE VII.—Rotation of Mannitol in Solution with 6.00 per cent. of Borax and 0.9 per cent. of Glucose.

| Conditions : <i>Present per 100 c.c. of Solution.</i> | | | | Polarimeter Tube. | | | |
|--|---------------------------------|----------------|---------|---------------------|---------------------------------|----------------|--------|
| Glucose, 0.900 gm. = 25 c.c. of a 3.6 per cent. solution. | | | | 4 dm. | | | |
| Na ₂ B ₄ O ₇ . 10H ₂ O 6.00 gm. | | | | Temperature, 20° C. | | | |
| Test No. | Gm. Mannitol per 100 c.c. | °Rotation. | | Test No. | Gm. Mannitol per 100 c.c. | °Rotation. | |
| | | Mercury Light. | | | | Mercury Light. | |
| | | Yellow. | Green. | | | Yellow. | Green. |
| 1 | 0.0 | — 0.009 | — 0.011 | 15 | 1.8 | 2.888 | 3.262 |
| 2 | 0.1 | + 0.130 | + 0.147 | 16 | 2.0 | 3.172 | 3.557 |
| 3 | 0.2 | 0.331 | 0.358 | 17 | 2.2 | 3.427 | 3.890 |
| 4 | 0.3 | 0.547 | 0.618 | 18 | 2.4 | 3.682 | 4.146 |
| 5 | 0.4 | 0.698 | 0.773 | 19 | 2.6 | 3.925 | 4.411 |
| 6 | 0.5 | 0.863 | 0.972 | 20 | 2.8 | 4.150 | 4.650 |
| 7 | 0.6 | 1.037 | 1.152 | 21 | 3.0 | 4.312 | 4.882 |
| 8 | 0.7 | 1.218 | 1.360 | 22 | 3.2 | 4.587 | 5.143 |
| 9 | 0.8 | 1.380 | 1.543 | 23 | 3.4 | 4.792 | 5.352 |
| 10 | 0.9 | 1.547 | 1.745 | 24 | 3.6 | 4.972 | 5.563 |
| 11 | 1.0 | 1.703 | 1.897 | 25 | 3.8 | 5.168 | 5.782 |
| 12 | 1.2 | 2.027 | 2.278 | 26 | 4.0 | 5.268 | 5.938 |
| 13 | 1.4 | 2.312 | 2.603 | 27 | 4.5 | 5.620 | 6.370 |
| 14 | 1.6 | 2.610 | 2.923 | 28 | 5.0 | 5.957 | 6.718 |
| Tests in which the glucose was weighed out { | | | | 29 | 1.0 | 1.704 | 1.896 |
| | | | | 30 | 2.0 | 3.169 | 3.561 |

$$\text{Ratio : } \frac{\text{Total Rotations by Green Light}}{\text{Total Rotations by Yellow Light}} = 1.1235.$$

A curve was drawn from the above results and smoothed out. From the smoothed curve a table was drawn up showing gm. mannitol for every 0.1° , up to 4.5° for the yellow light and 5.1° for the green light.

TABLE VIII.—Connecting $^\circ$ Rotation, with Mercury Yellow and Green Lights, of Mannitol in Solutions containing 6.00 per cent. of Borax and 0.9 per cent. of Glucose.

| °Rotation | Gm. Mannitol per 100 c.c. | | | | °Rotation | Gm. Mannitol per 100 c.c. | | | |
|-----------|---------------------------|-------|--------------|-------|-----------|---------------------------|-------|--------------|-------|
| | Yellow Light. | | Green Light. | | | Yellow Light. | | Green Light. | |
| | | Δ0.1° | | Δ0.1° | | | Δ0.1° | | Δ0.1° |
| 0.0 | 0.014 | 0.060 | 0.013 | 0.054 | 2.6 | 1.587 | 0.070 | 1.392 | 0.060 |
| 0.1 | 0.074 | 0.057 | 0.067 | 0.051 | 2.7 | 1.657 | 0.071 | 1.452 | 0.060 |
| 0.2 | 0.131 | 0.056 | 0.118 | 0.051 | 2.8 | 1.728 | 0.072 | 1.513 | 0.061 |
| 0.3 | 0.187 | 0.056 | 0.169 | 0.050 | 2.9 | 1.800 | 0.073 | 1.574 | 0.062 |
| 0.4 | 0.243 | 0.055 | 0.219 | 0.049 | 3.0 | 1.873 | 0.074 | 1.636 | 0.063 |
| 0.5 | 0.298 | 0.055 | 0.268 | 0.049 | — | — | — | — | — |
| 0.6 | 0.353 | 0.055 | 0.317 | 0.049 | 3.1 | 1.947 | 0.074 | 1.699 | 0.064 |
| 0.7 | 0.408 | 0.056 | 0.366 | 0.049 | 3.2 | 2.021 | 0.076 | 1.763 | 0.064 |
| 0.8 | 0.464 | 0.057 | 0.415 | 0.050 | 3.3 | 2.097 | 0.077 | 1.827 | 0.065 |
| 0.9 | 0.521 | 0.057 | 0.465 | 0.050 | 3.4 | 2.174 | 0.079 | 1.892 | 0.066 |
| 1.0 | 0.578 | 0.057 | 0.515 | 0.051 | 3.5 | 2.253 | 0.080 | 1.958 | 0.066 |
| 1.1 | 0.635 | 0.057 | 0.566 | 0.051 | 3.6 | 2.333 | 0.081 | 2.024 | 0.068 |
| 1.2 | 0.692 | 0.058 | 0.617 | 0.051 | 3.7 | 2.414 | 0.082 | 2.092 | 0.069 |
| 1.3 | 0.750 | 0.059 | 0.668 | 0.051 | 3.8 | 2.496 | 0.084 | 2.161 | 0.069 |
| 1.4 | 0.809 | 0.061 | 0.719 | 0.052 | 3.9 | 2.580 | 0.086 | 2.230 | 0.071 |
| 1.5 | 0.870 | 0.061 | 0.771 | 0.053 | 4.0 | 2.666 | 0.088 | 2.301 | 0.072 |
| 1.6 | 0.931 | 0.062 | 0.824 | 0.054 | 4.1 | 2.754 | 0.090 | 2.373 | 0.072 |
| 1.7 | 0.993 | 0.062 | 0.878 | 0.054 | 4.2 | 2.844 | 0.092 | 2.445 | 0.074 |
| 1.8 | 1.055 | 0.064 | 0.932 | 0.055 | 4.3 | 2.936 | 0.093 | 2.519 | 0.075 |
| 1.9 | 1.119 | 0.064 | 0.987 | 0.056 | 4.4 | 3.029 | 0.096 | 2.594 | 0.077 |
| 2.0 | 1.183 | 0.066 | 1.043 | 0.056 | 4.5 | 3.125 | — | 2.671 | 0.078 |
| 2.1 | 1.249 | 0.066 | 1.099 | 0.057 | 4.6 | — | — | 2.749 | 0.080 |
| 2.2 | 1.315 | 0.067 | 1.156 | 0.058 | 4.7 | — | — | 2.829 | 0.082 |
| 2.3 | 1.382 | 0.068 | 1.214 | 0.059 | 4.8 | — | — | 2.911 | 0.083 |
| 2.4 | 1.450 | 0.068 | 1.273 | 0.059 | 4.9 | — | — | 2.994 | 0.085 |
| 2.5 | 1.518 | 0.069 | 1.332 | 0.060 | 5.0 | — | — | 3.079 | 0.087 |
| 2.6 | 1.587 | 0.070 | 1.392 | 0.060 | 5.1 | — | — | 3.166 | — |

These figures apply to a 4 dm. tube.

TABLE IX.—Rotation of Mannitol in Solution with 6.00 gm. Borax and 0.5 gm. Glucose per 100 c.c. A 4 dm. tube was used.

| Gm. Mannitol. | °Rotation. | | Gm. Mannitol. | °Rotation. | |
|---------------|-------------------|------------------|---------------|-------------------|------------------|
| | Hg. Yellow Light. | Hg. Green Light. | | Hg. Yellow Light. | Hg. Green Light. |
| 0.0 | — 0.115 | — 0.134 | | | |
| 0.1 | + 0.090 | + 0.103 | 1.2 | 1.802 | 2.023 |
| 0.2 | 0.246 | 0.305 | 1.4 | 2.062 | 2.327 |
| 0.3 | 0.416 | 0.457 | 1.6 | 2.328 | 2.629 |
| 0.4 | 0.538 | 0.647 | 1.8 | 2.594 | 2.917 |
| 0.5 | 0.742 | 0.819 | 2.0 | 2.880 | 3.221 |
| 0.6 | 0.912 | 1.005 | 2.2 | 3.082 | 3.469 |
| 0.7 | 1.060 | 1.165 | 2.4 | 3.328 | 3.731 |
| 0.8 | 1.208 | 1.345 | 2.6 | 3.520 | 3.961 |
| 0.9 | 1.340 | 1.509 | 2.8 | 3.722 | 4.189 |
| 1.0 | 1.494 | 1.683 | 3.0 | 3.926 | 4.419 |

A smooth curve was drawn from the above results, and from the curve Table X was deduced, showing gm. mannitol corresponding to each 0.1° rotation.

SECTION B.—CHEMICAL METHODS FOR THE ESTIMATION OF MANNITOL.

The first chemical method tried was the precipitation of the mannitol as the tribenzoyl-acetal compound (MEUNIER, 1888). Glucose and other reducing sugars were believed not to interfere in any way in the precipitation of this derivative. Experiments with varying conditions of precipitation, however, gave irregular results which were also low—60 to 75 per cent. of theoretical. This method was therefore abandoned. An attempt to use *p*-nitrobenzoyl chloride as a precipitant was equally unsuccessful.

The acetylation method used in glycerol analysis was then tried, first of all on pure mannitol. The details of the test as applied to glycerol were followed as closely as possible, but filtration after acetylation was impossible because the mannitol hexa-acetate, if present in sufficient quantity, separated and would not pass the filter paper. The hexa-acetate on cooling formed a hard crystalline mass, which was broken up with a flat-ended glass rod previous to the neutralising operation. The results of the tests and conditions observed as to times and quantities are shown in Table XI.

TABLE X.—Connecting °Rotation, with Mercury Yellow and Green Lights, of Mannitol in Solutions containing 6.00 per cent. of Borax and 0.5 per cent. of Glucose.

| °Rotation | Gm. Mannitol per 100 c.c. | | | | °Rotation. | Gm. Mannitol per 100 c.c. | | | |
|-----------|---------------------------|-------|--------------|-------|------------|---------------------------|-------|--------------|-------|
| | Yellow Light. | | Green Light. | | | Yellow Light. | | Green Light. | |
| | | Δ0.1° | | Δ0.1° | | | Δ0.1° | | Δ0.1° |
| −0.1 | 0.005 | 0.056 | 0.012 | 0.049 | 2.2 | 1.495 | 0.074 | 1.318 | 0.064 |
| 0.0 | 0.061 | 0.057 | 0.061 | 0.050 | 2.3 | 1.569 | 0.076 | 1.382 | 0.064 |
| +0.1 | 0.118 | 0.058 | 0.111 | 0.052 | 2.4 | 1.645 | 0.076 | 1.446 | 0.066 |
| 0.2 | 0.176 | 0.059 | 0.163 | 0.052 | 2.5 | 1.721 | 0.077 | 1.512 | 0.066 |
| 0.3 | 0.235 | 0.060 | 0.215 | 0.053 | 2.6 | 1.798 | 0.078 | 1.578 | 0.067 |
| 0.4 | 0.295 | 0.061 | 0.268 | 0.054 | 2.7 | 1.876 | 0.081 | 1.645 | 0.068 |
| 0.5 | 0.356 | 0.062 | 0.322 | 0.055 | 2.8 | 1.957 | 0.083 | 1.713 | 0.069 |
| 0.6 | 0.418 | 0.062 | 0.377 | 0.055 | 2.9 | 2.040 | 0.084 | 1.782 | 0.069 |
| 0.7 | 0.480 | 0.064 | 0.432 | 0.055 | 3.0 | 2.124 | 0.085 | 1.851 | 0.071 |
| 0.8 | 0.544 | 0.064 | 0.487 | 0.057 | 3.1 | 2.209 | 0.087 | 1.922 | 0.073 |
| 0.9 | 0.608 | 0.064 | 0.544 | 0.057 | 3.2 | 2.296 | 0.090 | 1.995 | 0.074 |
| 1.0 | 0.672 | 0.064 | 0.601 | 0.057 | 3.3 | 2.386 | 0.093 | 2.069 | 0.075 |
| 1.1 | 0.736 | 0.065 | 0.658 | 0.057 | 3.4 | 2.479 | 0.095 | 2.144 | 0.076 |
| 1.2 | 0.801 | 0.066 | 0.715 | 0.058 | 3.5 | 2.574 | 0.096 | 2.220 | 0.078 |
| 1.3 | 0.867 | 0.067 | 0.773 | 0.058 | 3.6 | 2.670 | 0.096 | 2.298 | 0.080 |
| 1.4 | 0.934 | 0.067 | 0.831 | 0.059 | 3.7 | 2.768 | 0.099 | 2.378 | 0.082 |
| 1.5 | 1.001 | 0.068 | 0.890 | 0.059 | 3.8 | 2.867 | 0.101 | 2.460 | 0.084 |
| 1.6 | 1.069 | 0.068 | 0.949 | 0.060 | 3.9 | 2.968 | 0.103 | 2.544 | 0.085 |
| 1.7 | 1.137 | 0.070 | 1.009 | 0.060 | 4.0 | 3.071 | — | 2.629 | 0.086 |
| 1.8 | 1.207 | 0.070 | 1.069 | 0.061 | 4.1 | — | — | 2.715 | 0.088 |
| 1.9 | 1.277 | 0.072 | 1.130 | 0.062 | 4.2 | — | — | 2.803 | 0.089 |
| 2.0 | 1.349 | 0.072 | 1.192 | 0.064 | 4.3 | — | — | 2.892 | 0.091 |
| 2.1 | 1.421 | 0.074 | 1.254 | 0.064 | 4.4 | — | — | 2.983 | — |
| 2.2 | 1.495 | 0.074 | 1.318 | 0.064 | — | — | — | — | — |

These figures apply to a 4 dm. tube.

Notes on Table XI.

Results given in tests 1–7 where alkali hydrolysis was continued for 20 minutes, are very good, but on investigating the consistency possible by the method, results were found to vary within 1 per cent. in tests 11–20. All precautions were taken against carbon dioxide, and no reason was found for this somewhat large variation. The melting point of the purified material used was correct, 166°C. , and further purification did not change the melting point. Reducing the quantity of reagents in the acetylation did not seem to have any effect on the result, variations being of the same order as was found with the usual quantities of reagents.

TABLE XI.—Application of Acetylation Method to Mannitol.

| Test No. | Gm. Mannitol. | Gm. Anhydrous Sodium Acetate. | c.c. Acetic Anhydride. | NaOH Hydrolysis in minutes. | Result as Percentage Purity. | Remarks. |
|----------|---------------|-------------------------------|------------------------|-----------------------------|------------------------------|---|
| 1 | 1.1 | 3.0 | 7.5 | 20 | 99.9 | It is advisable to continue the NaOH hydrolysis for 20 min. after the solution has been brought to boiling point. |
| 2 | 0.9 | 3.0 | 7.5 | 20 | 100.2 | |
| 3 | 0.8 | 3.0 | 7.5 | 20 | 99.8 | |
| 4 | 0.5 | 3.0 | 7.5 | 10 | 99.1 | |
| 5 | 0.6 | 3.0 | 7.5 | 15 | 99.6 | |
| 6 | 0.5 | 3.0 | 7.5 | 20 | 100.0 | |
| 7 | 1.1 | 3.0 | 7.5 | 20 | 100.0 | |
| 8 | 0.5 | 1.5 | 4 | 20 | 100.6 | Carried out to find the effect of reducing the quantities of reagents. |
| 9 | 0.5 | 1.5 | 4 | 20 | 99.4 | |
| 10 | 0.5 | 1.5 | 7.5 | 20 | 99.2 | |
| 11 | 1.0 | 3 | 7.5 | 20 | 100.1 | Tests 11-20 carried out to find what consistency of results is to be expected by the method. |
| 12 | 1.1 | 3 | 7.5 | 20 | 100.0 | |
| 13 | 0.9 | 3 | 7.5 | 20 | 99.4 | |
| 14 | 1.1 | 3 | 7.5 | 20 | 99.6 | |
| 15 | 1.2 | 3 | 7.5 | 20 | 99.1 | |
| 16 | 0.9 | 3 | 7.5 | 20 | 99.5 | |
| 17 | 1.1 | 3 | 7.5 | 20 | 99.2 | |
| 18 | 1.0 | 3 | 7.5 | 20 | 99.6 | |
| 19 | 1.0 | 3 | 7.5 | 20 | 99.5 | |
| 20 | 1.0 | 3 | 7.5 | 20 | 99.8 | |
| | | Mean of 20 results ... | ... | ... | 99.6 | Heated with acetic anhydride for 2 hours. Heated with acetic anhydride for 1½ hours. |

SECTION C.—ANALYSIS OF SYNTHETIC MIXTURES OF MANNITOL AND GLUCOSE BY THE POLARIMETER AND ACETYLATION METHODS, BY A METHOD INVOLVING REMOVAL OF GLUCOSE BY YEAST FERMENTATION.

Fermentation solutions containing mannitol usually contain carbohydrate residues. This, in the case under examination, is, of course, glucose. In devising a method for the estimation of mannitol in fermentation solutions measures must therefore be taken either to remove the carbohydrate before estimating the mannitol, or to ensure that the carbohydrate, if not removed, will not interfere with the estimation of the mannitol. It was realized that it was desirable, because of the close similarity in chemical composition between mannitol and the hexoses, to remove the carbohydrate first. The only satisfactory method of doing this is by fermentation with a pure culture of yeast—preferably of *Saccharomyces cerevisiæ*. This yeast not only destroys completely all the sugars likely to be met with in fermentation work, *e.g.*, glucose, fructose, sucrose and maltose, but has no action on mannitol. The experiments carried out to

define conditions for the estimation by this method of mannitol and glucose dissolved in CZAPEK-DOX solution are described on pp. 188-198. Unfortunately this method, while giving very satisfactory results, is time-consuming, since it is essential to work with sterile solutions and a pure culture of yeast. Hence conditions were worked out for the estimation of mannitol in presence of glucose which did not involve the removal of this sugar. These are described on pp. 198-200.

The polarimeter and acetylation tests were therefore applied to synthetic mixtures of mannitol and glucose. Known amounts of these substances were dissolved in 350 c.c. quantities of the usual CZAPEK-DOX medium, containing either 0.2 per cent. of NaNO_3 , or 0.2 per cent. of NH_4NO_3 .

The flasks containing the solutions of mannitol and glucose in CZAPEK-DOX NH_4NO_3 solution were plugged with cotton wool and sterilized by steaming at 100°C . for 20 minutes on three consecutive days. A quantity of a pure culture of *Saccharomyces cerevisiae*, sufficient to ferment all the carbohydrate present, was then added and allowed to ferment for two to three days at a temperature of 30°C . The pure culture was prepared by sowing sterile beer wort agar in Roux bottles with a pure culture of *S. cerevisiae* and emulsifying the growth after three days in sterile physiological saline solution (0.86 per cent. sodium chloride in distilled water). The small amount of beer wort adhering to the yeast cells was removed by three washings under sterile conditions with the saline solution and separation of the cells by centrifuging after each washing. After fermentation of the sugar, the yeast was separated by filtration. The filtrate was made neutral to bromthymol blue and treated with 10 c.c. of a 20 per cent. solution of normal lead acetate. The precipitate required about two hours to settle, after which it was removed by filtration. The filtrate was again neutralised to bromthymol blue, and then treated with 10 c.c. of a 10 per cent. basic lead acetate solution. The precipitate was allowed to settle, which sometimes required an overnight period, after which it was removed by filtration and the filtrate treated with sulphuretted hydrogen. After the lead sulphide had settled it was removed by filtration. The filtrate was evaporated under reduced pressure to a suitable volume and finally made up to a standard volume of 250 c.c. Aliquot portions were transferred to 250 c.c. round-bottomed flasks and evaporated to dryness by means of a current of hot air. The flask containing the residue was then placed in a boiling water oven for an hour, after which the residue was acetylated. Results of these tests are shown in Table XII. Polarimeter results are also given. In carrying out the polarimeter work, the rotation of the standard solution was first taken to determine whether any glucose had survived the yeast treatment. Then 150 c.c. of the standard solution were made up to 250 c.c. containing 15.00 gm. of borax, that is, 6 per cent. The mercury yellow light was used.

The polarimeter results were considered satisfactory. The high results obtained by acetylation are almost certainly due to the presence of glycerol formed from glucose by fermentation during the removal of the carbohydrate by yeast. Thus, while the presence of 5 per cent. of glycerol would only effect the percentage results shown by

TABLE XII.—Estimation of Mannitol in Synthetic Mannitol-Glucose Mixtures in CZAPEK-DOX Solution.

| | Test No. | Mannitol Gm. | Glucose Gm. | After Acting on Mixture with Yeast. | | | | | |
|-------------|----------|--------------|-------------|-------------------------------------|-----|-----------------|---|----------------------------------|--------------------------------|
| | | | | Glucose. | | Mannitol. | | | |
| | | | | °Rota- tion. | Gm. | °Rota- tion. | Gm. per 100 c.c. Borax Solution. | Total Mannitol Accounted for. | |
| | | | | | | | | Gm. | Percent- age Re- covery. |
| Polarimeter | 1 | 8.7505 | 8.7507 | 0.00 | Nil | 2.656 | ≡ 2.071 | 8.629 | 98.9 |
| | 2 | 8.7504 | 8.7510 | 0.00 | Nil | 2.645 | ≡ 2.060 | 8.584 | 98.1 |
| Acetylation | 1 | 8.7505 | 8.7507 | — | — | — | Test 1 { | (i) 9.107 | 104.1 |
| | | | | | | | | (ii) 9.036 | 103.3 |
| | 2 | 8.7504 | 8.7510 | — | — | — | Test 2 { | (i) 9.234 | 105.5 |
| | | | | | | | | (ii) 9.320 | 106.5 |

the polarimeter by 0.2–0.3 per cent., the full 5 per cent. would be shown in the acetylation figures. The formation of glycerol in the fermentation of glucose by yeast is now a well-known fact. BUCHNER and MEISENHEIMER (1905) obtained an average figure of 3.8 per cent. of glycerol on the weight of sugar fermented. In order to test the point, a third test was carried out in which glucose was present without mannitol. 8.75 gm. of glucose were treated with yeast and the final solution of the products obtained made up to 250 c.c. The rotation of this solution was $+0.033^\circ$ using the mercury yellow light, equivalent to 0.0375 gm. of glucose in 250 c.c., which is negligible in comparison with the acetylation value of the solution which was found to be 0.49 and 0.42 gm. glycerol in 250 c.c., equivalent to about 5 per cent. of the glucose used. The difference between the glycerol results suggests that some glycerol is lost in the evaporation with hot air.

An attempt was made to obviate the errors introduced into the acetylation estimation by the formation of glycerol from glucose during fermentation. This was effected by evaporating an aliquot portion of the solution as usual and extracting the glycerol with absolute alcohol, ether being then added in order to precipitate any mannitol taken up by the alcohol. Hence it became necessary to determine whether the loss of mannitol by extraction is appreciable. The solubility of mannitol in boiling absolute alcohol was determined by boiling 0.5 gm. of the substance with 200 c.c. of the solvent under reflux. The last few particles required about half an hour to dissolve. 0.1 gm.

quantities were added when solution of the previous quantity was complete. In this way 0.9 gm. were dissolved after six to seven hours' boiling. A further 0.1 gm. did not dissolve on prolonged boiling. The solubility of mannitol in boiling absolute alcohol may therefore be taken as 0.45 gm. per 100 c.c. The solubility of mannitol in cold absolute alcohol and in a mixture of 1 part of absolute alcohol with 4 parts of sodium-dried ether was also determined. 0.05 gm. of mannitol was dissolved in absolute alcohol, with gentle heating. When the solution was allowed to cool mannitol settled out and was separated by filtration. 50 c.c. of the filtrate were evaporated and found to contain 0.0081 gm. of mannitol. The solubility of mannitol in absolute alcohol at ordinary temperatures is therefore 0.016 gm. per 100 c.c. 50 c.c. of the filtrate were treated with 200 c.c. of dry ether and the mixture allowed to stand for half an hour. The contents of the flask were then filtered, and 200 c.c. of the filtrate were evaporated. The residue weighed 0.0052 gm. The solubility of mannitol in a 1 : 4 alcohol-ether mixture is therefore 0.0026 gm. per 100 c.c., *i.e.*, only one-sixth of that in alcohol alone.

The first attempts to remove glycerol were carried out as follows :—

25 c.c., *i.e.*, one-tenth of the total fermented solution, were evaporated to dryness in a flask by means of hot air and then heated at 100° C. in an oven for an hour. The flask was cooled, 50 c.c. of absolute alcohol were added and the residue was extracted by boiling under reflux for half an hour. The flask was then cooled, 200 c.c. of sodium-dried ether were added, and the contents of the flask were allowed to settle. The liquid portion was then separated by filtration. The residue and the filter paper were washed with small quantities of 1 : 4 alcohol-ether mixture. The solid on the filter paper was washed back with hot water into the extraction flask, the contents of which were again evaporated to dryness. The residue thus obtained, called residue No. 1 in Table XIII, was acetylated. The filtrate from the first extraction was evaporated to dryness and re-extracted, the quantities of alcohol and ether used being 5 c.c. and 20 c.c. respectively. Further extractions were carried out in a similar manner. The results of a number of tests are given in Table XIII, in which all acetylation results are calculated to percentage of mannitol.

The figures in Table XIII show that the polarimeter could be relied upon to give results within 97–100 per cent. of the mannitol used, without any correction for glycerol. The amount of glucose left after the treatment with yeast is small and the correction on the mannitol figure for the amounts actually found did not exceed 1 per cent. The corrections applied in Table XIII are approximate. If a more accurate correction were required, a very large amount of work on synthetic mixtures of mannitol, glucose and glycerol would be required. In tests 4 and 5, no direct reading of the final solutions without borax was obtained as the solutions were too dense optically. The glycerol content depends on the original glucose content to a great extent. This is evident even although results for any one test vary considerably among themselves, due probably to the volatility of glycerol.

TABLE XIII.—Estimation of Mannitol in Synthetic Mannitol-Glucose Mixtures by Polarimeter and Acetylation Methods, modified as described immediately above by Alcohol-Ether Extraction of Glycerol before Acetylation.

| Test No. | 4. | | | 5. | | 6. | | | 7. | | | 8. | | | |
|---|---------|-------|-------|--------|-------|--------|-------|-------|---------|-------|-------|---------|-------|-------|--|
| Mannitol taken ... gm. | 10.0005 | | | 9.9997 | | 6.0016 | | | 3.0020 | | | 6.0017 | | | |
| Glucose taken ... „ | 6.0011 | | | 6.0343 | | 9.9958 | | | 12.9900 | | | 10.0050 | | | |
| Final solution made up to ... c.c. | 250 | | | 250 | | 250 | | | 250 | | | 250 | | | |
| Rotation of final solution without borax ... | — | | | — | | 0.142° | | | 0.357° | | | 0.060° | | | |
| Volume of final solution made up to 250 c.c. with 15 gm. (6 per cent.) of borax... c.c. | 150 | | | 125 | | 150 | | | 150 | | | 150 | | | |
| Rotation of borax solution ... | 2.944° | | | 2.549° | | 1.924° | | | 1.004° | | | 1.934° | | | |
| Total mannitol accounted for, uncorrected... gm. | 9.944 | | | 9.838 | | 5.911 | | | 2.913 | | | 5.948 | | | |
| Mannitol accounted for— | | | | | | | | | | | | | | | |
| 1. Uncorrected percent. | 99.4 | | | 98.4 | | 98.5 | | | 97.1 | | | 99.1 | | | |
| 2. Corrected approximately for glycerol content. per cent. | 99.6 | | | 98.7 | | 98.8 | | | 99.0 | | | 99.5 | | | |
| 3. Recorrected approximately for glucose content. per cent. | — | | | — | | 98.1 | | | 98.2 | | | 99.1 | | | |
| <i>Acetylation results—</i> | | | | | | | | | | | | | | | |
| Test No.— | (i) | (ii) | (iii) | (i) | (ii) | (i) | (ii) | (iii) | (i) | (ii) | (iii) | (i) | (ii) | (iii) | |
| 1st residue ... | 99.0 | 98.4 | 97.1 | 104.7 | 97.1 | 109.8 | 99.8 | 96.2 | 131.5 | 102.6 | 78.1 | 106.1 | 93.8 | 95 | |
| 2nd residue ... | — | 1.7 | 4.4 | — | 1.2 | — | 7.6 | 5.2 | — | 13.3 | 33.7 | — | 9.7 | 9.6 | |
| 3rd residue ... | — | 3.3 | 3.3 | — | 2.6 | — | 3.2 | 0.6 | — | 6.3 | 23.3 | — | 3.8 | 0.6 | |
| 4th residue ... | — | — | — | — | — | — | — | 2.2 | — | — | — | — | — | 3.0 | |
| <i>Total acetyl value—</i> | | | | | | | | | | | | | | | |
| As Mannitol per cent. | 99.0 | 103.4 | 104.8 | 104.7 | 100.9 | 109.8 | 110.6 | 104.2 | 131.5 | 122.2 | 135.1 | 106.1 | 107.3 | 108.6 | |

Two further tests were carried out as follows:—

The synthetic mixtures were treated as before, except that, after the removal of the lead, the solution was evaporated to dryness in a Claisen flask. After the residue had been dried as far as possible *in vacuo* at 60° C., in the Claisen flask, successive quantities of absolute alcohol were added and distilled off, to help to free the residue from water. 40 c.c. of alcohol were then added and the contents of the flask boiled under reflux for one hour. The flask was cooled and 200 c.c. of sodium-dried ether were added. The ether-alcohol mixture was filtered off, and residue and filter paper were washed with 50 c.c. of ether-alcohol mixed in the proportion 4:1. The solid on the filter paper was transferred back to the Claisen flask with hot water. The alcohol and ether in the flask were removed by distillation and the mannitol was finally transferred to a standard 250 c.c. flask. The tests carried out on this material are shown in Table XIV under the heading "Solid Residue." The ether-

alcohol filtrate was evaporated until all solvent was removed. The residue is referred to in Table XIV as "Residue ex Filtrate." This solution was tested without and with borax in the polarimeter, and also by acetylation. In the tests on the "Solid Residue," different quantities of the solution were used in preparing the polarimeter solutions, to test whether the residual CZAPEK-DOX salts had any effect on the rotation.

TABLE XIV.—Estimation of Mannitol in Synthetic Mixtures of Mannitol and Glucose, by Polarimeter and Acetylation Methods. The solution under test, after the yeast and subsequent treatments, was evaporated to dryness and the whole of the residue extracted once with alcohol, to remove glycerol. The mercury yellow light was used.

| Test No. | Weight taken, gm. | | Solid Residue. | Residue ex Filtrate. |
|----------|-------------------|----------|---|--|
| | Mannitol. | Glucose. | Made up to 250 c.c., Soln. A. | Made up to 100 c.c., Soln. D. |
| 9 | 8.711 | 8.740 | <p><i>Acetylation result</i>: 99.7 per cent. Mannitol</p> <p><i>Polarimeter results</i>—</p> <p>(i) 100 c.c. A. evaporated, 6 gm. borax added, made up to 100 c.c., gave rotation $3.857^\circ \equiv 3.452$ gm. mannitol = 99.1 per cent. of theory</p> <p>(ii) 25 c.c. A + 3 gm. borax made up to 50 c.c. gave rotation $2.288^\circ \equiv 1.728$ gm. mannitol = 99.2 per cent. of theory</p> <p>(iii) Rotation of Soln. A without borax, zero</p> | <p><i>Acetylation result</i>: 1.3 per cent. as mannitol.</p> <p><i>Polarimeter results</i>—</p> <p>(i) Rotation without borax, zero.</p> <p>(ii) 50 c.c. of D. evaporated, then made up with 3 gm. of borax, gave zero rotation.</p> |
| 10 | 8.880 | 8.775 | <p><i>Acetylation results</i>—</p> <p>(i) 100.3 } mean = 100.0</p> <p>(ii) 99.6 }</p> <p><i>Polarimeter results</i>—</p> <p>(i) 125 c.c. Soln. A. evapd., 6 gm. borax added, made up to 100 c.c., gave Soln. B rotation $4.375^\circ \equiv 4.283$ gm. mannitol = 96.5 per cent. of theory</p> <p>(ii) 25 c.c. A + 3 gm. borax made up to 50 c.c., gave rotation $2.295^\circ \equiv 1.735$ gm. = 97.7 per cent.</p> <p>(iii) 25 c.c. B + 4.5 gm. borax made up to 100 c.c., gave rotation $1.525^\circ \equiv 1.097$ gm. = 98.8 per cent.</p> <p>(iv) Rotation of A without borax, zero</p> | <p><i>Acetylation result</i>—</p> <p>1.8 per cent. as mannitol.</p> <p><i>Polarimeter results</i>—</p> <p>Same conditions as above, rotation zero in both cases.</p> |

The treatment given in the tests in Table XIV appears to have been an improvement on the previous method. The acetylation results in both tests are good and the residue seems to have been well freed from glycerol. In addition, mannitol seems to be absent in the residue from the filtrate. In the polarimeter tests, it appears to be inadvisable to use too large a quantity of the standard solution in the preparation of the borax solution. The concentrations used in preparing the solutions in Table XIII appear to have been suitable as judged by results. Another experiment to test the point was made in the same way as Nos. 9 and 10 in Table XIV. The results of the test, in which polarimetric work only was done, are given in Table XV.

TABLE XV.—Effect of Concentration in the Polarimeter Solutions.
Test No. 11.

| Weight taken, gm. | | Solid Residue made up to 250 c.c. gave Soln. A. |
|-------------------|----------|---|
| Mannitol. | Glucose. | |
| 8.709 | 8.748 | (i) 120 c.c. A + 6 gm. borax gave 100 c.c. Soln. B. Rotation of B, $4.100^\circ \equiv 3.806$ gm. mannitol = 91.1 per cent. of theory. (ii) 25 c.c. A + 3 gm. borax gave 50 c.c. Soln. C. Rotation of C, $2.260^\circ \equiv 1.704$ gm. mannitol = 97.8 per cent. of theory. |

These results bear out the conclusion drawn from Table XIV, that too much of the solution of the "Solid Residue" should not be used in preparing the polarimeter solution. As before, the solution of the solid residue was neutralised before the borax was added.

An attempt was therefore made to find an explanation of the low results obtained with concentrated solutions. These low results may be due to

- (a) the effect of the sodium acetate produced by treatment of the fermented solution with lead acetate,
- (b) the acidity of the CZAPEK-DOX salts,
- (c) the effect of heating mannitol in borax solution, or,
- (d) the combined effect of the residual CZAPEK-DOX salts.

The effect of each of these factors was tested in turn.

(a) It was found by calculation that 5 per cent. of sodium acetate in the polarimeter solution would cover the range of salt likely to be present in fermentation solutions. Tests were therefore carried out on solutions containing 2.015 per cent. mannitol, 6 per cent. borax and 0, 1 and 5 per cent. of sodium acetate. The results are given in Table XVI.

TABLE XVI.—Effect of Sodium Acetate in the Polarimeter Test.

| Test No. | Gm. per 100 c.c. of Solution. | | | | |
|----------|-------------------------------|--------|-----------------|------------------|-----------------|
| | Mannitol. | Borax. | Sodium acetate. | °Rotation Found. | °Rotation Calc. |
| 12 | 2·015 | 6·00 | Nil | 2·602 | 2·599 |
| 13 | 2·015 | 6·00 | 1·0 | 2·579 | 2·599 |
| 14 | 2·015 | 6·00 | 5·0 | 2·573 | 2·599 |

The differences found are thus not sufficient to account for the 9 per cent. difference shown in Table XV.

(b) The effect of acidity from CZAPEK-DOX salts was tried. A CZAPEK-DOX solution having forty times the usual salt concentration was prepared and filtered. 25 c.c. of the filtrate were used to prepare 100 c.c. of a mannitol solution containing 2·015 gm. mannitol and 6·00 gm. borax. The rotation fell from 2·599° to 2·230°, a difference of 0·369°. 25 c.c. of the filtrate were then made neutral to bromthymol blue, requiring 10 c.c. N/1 standard alkali and the neutralised solution was used in the preparation of a mannitol solution similar to the previous one. The rotation in this case was 2·540°, showing a decrease of 0·059°. The effect of neutral salts is therefore small compared with that of acid salts, and is much too small to account for the large difference found in test 11 of Table XV.

(c) The effect on rotation of heating mannitol with borax was tested by preparing two solutions in one of which the borax and mannitol were dissolved in the cold, in the other by heating on a boiling water bath for 30 minutes. The results of these tests were as follows:—

TABLE XVII.—Effect on Rotation of Heating Mannitol and Borax in Solution.

| Test No. | Mannitol. | Borax | Solution effected. | °Rotation. | | |
|----------|-----------|-------|------------------------|------------|-------|-------|
| | | | | Found. | Calc. | Diff. |
| | Gm. | Gm. | | | | |
| 15 | 2·960 | 6·00 | In cold | 3·458 | 3·475 | 0·017 |
| 16 | 3·088 | 6·00 | Over boiling water ... | 3·568 | 3·579 | 0·011 |

It seems from these figures that mannitol may be heated in borax solution without changing the rotation appreciably.

(d) Three more tests were carried out in each of which the products from the fermentation of three one-gram quantities of mannitol and glucose, each with normal

quantities of CZAPEK-DOX solution, were combined, giving three times the normal concentration of salts in the final solution used in the tests. The results of these three tests are given in Table XVIII.

TABLE XVIII.—Effect on Rotation of Three Times the Normal Concentration of CZAPEK-DOX Salts.

| Test No. | Weight Taken. | Solid Residue made up to 250 c.c. Solution A. | Residue ex Filtrate made up to 100 c.c. Solution D. |
|----------|--|--|---|
| 17 | Gm. <i>Mannitol</i> — (i) 1·017 (ii) 1·063 (iii) 1·052 Total 3·132 <i>Glucose</i> — (i) 1·010 (ii) 1·003 (iii) 1·003 Total 3·016 | <i>Acetylation results</i> — (i) 115·7 per cent. as mannitol. (ii) 117·0 per cent. as mannitol. <i>Polarimeter results</i> — (i) 125 c.c. A + 6 gm. borax gave 100 c.c. Solution B. Rotation of B, $2\cdot030^\circ \equiv 1\cdot506$ gm. mannitol. = 96·2 per cent. of theory. (ii) 25 c.c. of B + 4·5 gm. borax gave 100 c.c. Solution C. Rotation of C, $0\cdot575^\circ \equiv 0\cdot386$ gm. mannitol. = 98·6 per cent. of theory. | No extraction in Test 17. |
| 18 | <i>Mannitol</i> — 3·000 gm. <i>Glucose</i> — 3·000 gm. | <i>Acetylation results</i> — (i) 108·9 per cent. as mannitol. (ii) 109·1 per cent. as mannitol. <i>Polarimeter results</i> — Solutions prepared as in Test No. 17— (i) Rotation of B, $1\cdot990^\circ \equiv 1\cdot474$ gm. mannitol. = 98·3 per cent. of theory. (ii) Rotation of C, $0\cdot555^\circ \equiv 0\cdot372$ gm. mannitol. = 99·2 per cent. of theory. | <i>Acetylation result</i> — 2·5 per cent. as mannitol. <i>Polarimeter results</i> — Rotation zero, without and with borax. |
| 19 | <i>Mannitol</i> — 3·000 gm. <i>Glucose</i> — 3·000 gm. | <i>Acetylation results</i> — (i) 105·5 per cent. as mannitol. (ii) 102·1 per cent. as mannitol. <i>Polarimeter results</i> — Solutions prepared as in Test 17. (i) Rotation of B, $2\cdot000^\circ \equiv 1\cdot481$ gm. mannitol. = 98·7 per cent. of theory. (ii) Rotation of C, $0\cdot555^\circ \equiv 0\cdot372$ gm. mannitol. = 99·2 per cent. of theory. | <i>Acetylation results</i> — (i) 2·3 per cent. as mannitol. (ii) 2·0 per cent. as mannitol. <i>Polarimeter results</i> — Rotation zero, without and with borax. |

Notes on Table XVIII.

The accuracy of the polarimeter results with the lower concentrations is within ± 1 per cent., and with the higher concentrations, say $\pm 0\cdot5$ per cent., calculated on the percentage figures for mannitol. The greatest difference between the higher and

lower concentrations is in test 17 and amounts to 2.4 per cent., which is not nearly so great as the 9 per cent. difference shown in Table XV. The residual CZAPEK-Dox salts, therefore, do not seem to have a very marked effect, and polarimeter results are probably reliable if, say, 50 c.c. of the solution of the "Solid Residue" are made up to 100 c.c. with 6 gm. of borax.

Single extractions in tests 18 and 19 did not extract the glycerol so cleanly as in tests 9 and 10 of Table XIV. The effect of repeated extraction of the total residue is given later in this paper.

A few tests were carried out to determine the loss of glycerol in the evaporation of solutions by means of hot air, an essential step before the mannitol can be acetylated. The evaporation is carried out by immersing the flask containing the solution in a bath of boiling water. Hot air is blown over the surface of the solution, the air being heated by passing it through a long glass spiral submerged in boiling water. In each test, 50 c.c. of a solution containing 0.5002 gm. mannitol, and a known volume of a glycerol solution were evaporated in a round-bottomed acetylation flask in the usual way. The residue was further dried *in vacuo* overnight in a desiccator over sulphuric acid. The dried material was then acetylated in the usual way. The results of the tests are given in Table XIX.

TABLE XIX.—Loss of Glycerol during Evaporation of Solutions of Mannitol and Glycerol by means of Hot Air.

| Test No. | Weight taken, gm. | | Acetylation Value as Gm. Mannitol (c). | Difference (c — a). | Difference as Glycerol (d). | Glycerol lost (b — d). |
|----------|-------------------|---------------|--|---------------------|-----------------------------|------------------------|
| | Mannitol (a). | Glycerol (b). | | | | |
| 20 | 0.5002 | 0.2800 | 0.6804 | 0.1802 | 0.1822 | 0.0978 |
| 21 | 0.5002 | 0.1400 | 0.5693 | 0.0691 | 0.0699 | 0.0701 |
| 22 | 0.5002 | 0.0560 | 0.5282 | 0.0280 | 0.0283 | 0.0277 |

It will be seen that there is a distinct loss of glycerol.

In view of the incomplete extraction of glycerol shown in Table XVIII, two tests were carried out on solutions containing pure mannitol and glycerol only. The solutions were evaporated in hot air and the residue treated with absolute alcohol and sodium-dried ether as usual. The solid residue and the residue from the filtrate were tested by polarimeter and acetylation with the results given in Table XX.

Notes on Table XX.

The acetylation and the polarimeter results are both good, so that one extraction of the residue seems to have effectively removed the glycerol. The only difference between the original residues in Table XX and those in Table XVIII is that the

TABLE XX.—Effect of one Extraction with Alcohol of the Residue after Evaporation of a Solution of Pure Mannitol and Glycerol.

| Test No. | Weight Taken. | Solid Residue made up to 250 c.c. Solution A. | Residue ex Filtrate made up to 100 c.c. Solution D. |
|----------|--|--|---|
| 23 | Gm. Mannitol—6.2504 Glycerol—0.336 | <p><i>Acetylation result</i>— 99.6 per cent. as mannitol.</p> <p><i>Polarimeter results</i>— 125 c.c. A. evaporated + 6 gm. borax gave 100 c.c. Solution B. Rotation of B, $3.595^\circ \equiv 3.108$ gm. mannitol. = 99.4 per cent. of theory. 25 c.c. B + 4.5 gm. borax gave 100 c.c. Solution C. Rotation of C, $1.115^\circ \equiv 0.782$ gm. mannitol. = 100.1 per cent. of theory.</p> | <p><i>Acetylation results</i>— Found— (i) 0.130 gm. as glycerol. (ii) 0.088 gm. as glycerol. Glycerol added — 0.336 gm.</p> <p><i>Polarimeter results</i>— Rotation without and with borax, zero.</p> |
| 24 | Mannitol—6.2500 Glycerol—1.120 | <p><i>Acetylation result</i>— 98.4 per cent. as mannitol.</p> <p><i>Polarimeter results</i>— Solutions prepared as in test 23. Solution B gave rotation 3.595°. = 3.108 gm. mannitol. = 99.4 per cent. of theory. Solution C gave rotation 1.105°. = 99.2 per cent. of theory.</p> | <p><i>Acetylation results</i>— Found— (i) 0.811 gm. (ii) 0.712 gm. Glycerol added—1.120 gm.</p> |

latter were distinctly gummy in character, probably due to traces of yeast extractives, while the former were much more friable and hence more easily penetrated by the solvent. No mannitol was found in the residues from the filtrates.

Further Tests on Synthetic Mixtures in Sodium Nitrate Czapek-Dox Medium.

Previous work having been carried out with ammonium nitrate CZAPEK-DOX medium, two tests were now carried out with sodium nitrate in place of ammonium nitrate. The residues obtained after yeast treatment were extracted several times with alcohol in order to remove compounds of the glycerol type. The filtrates were combined and evaporated together. The results obtained are shown in Table XXI.

Notes on Table XXI.

The results in Table XXI do not differ from the preceding work. Test 26 shows a good separation of glycerol, but test 25 shows 2.5 per cent. of glycerol not removed even after three extractions. It is probably worth while, however, to make several extractions as glycerol has a depressing effect on the rotation of mannitol in borax solution.

TABLE XXI.—Results of Tests on Mixtures of Mannitol and Glucose in Sodium Nitrate CZAPEK-DOX Solution.

| Test No. | Weight Taken. | No. of Extractions. | Results on Solid Residue. | | | Results on Residue ex Filtrate. | | |
|----------|---|---------------------|--|------------------|---|---------------------------------|------------------|---|
| | | | Polarimeter. | | Acetylation. | Polarimeter. | | Acetylation. |
| | | | Borax. 6 per cent. | Borax, Nil. | | Borax. 6 per cent. | Borax, Nil. | |
| 25 | Gm. <i>Mannitol</i> 4.0227 <i>Glucose</i> 8.010 | 3 | 3.980 gm. = 98.9 per cent. of theory | Zero rotation | Per cent. (i) 102.0 (ii) 102.8 as mannitol | Per cent. 0.4 as mannitol | Zero rotation | Gm. (i) 0.150 (ii) 0.139 Mean = 3.5 per cent. as gly- cerol. |
| 26 | <i>Mannitol</i> 6.1620 <i>Glucose</i> 6.108 | 4 | 6.080 gm. = 98.7 per cent. of theory | Zero rotation | (i) 99.8 (ii) 100.2 as mannitol | 0.3 as mannitol | Zero rotation | (i) 0.226 (ii) 0.187 Mean = 3.5 per cent. as gly- cerol. |

SECTION D.—ANALYSIS OF METABOLISM SOLUTIONS CONTAINING MIXTURES OF MANNITOL AND GLUCOSE WITHOUT REMOVAL OF GLUCOSE.

The method of estimating mannitol in which glucose is first removed by means of a yeast fermentation is very time-consuming. Work was therefore undertaken to devise a method of estimating mannitol in presence of glucose using the results given in Tables II, VIII and X, and a solution derived from actual experiment was tested before and after yeast treatment. This solution was an average sample of the metabolism solution taken from a large laboratory scale experiment with a white species of *Aspergillus*, Ac. 55 (see Part IX, p. 170). This mould had been cultivated on a CZAPEK-DOX sodium nitrate solution ($pH=7.4$) containing 5 per cent. of glucose, and the metabolism solution remaining at the end of the fermentation was treated with lead acetate to remove organic impurities. The filtrate, which contained lead and is referred to in Table XXII as the "original solution," was used for analysis.

In the tests, a known amount of this solution was taken and the lead removed by means of sulphuretted hydrogen. The remainder of the treatment and the results of the tests are given in Table XXII.

TABLE XXII.

| TEST 1. | | | | | | | | | |
|---|-----|-----|-----|-----|-----|-----|-----|-----|-----------------|
| Glucose present in original solution— | | | | | | | | | |
| 1. By polarimeter | ... | ... | ... | ... | ... | ... | ... | ... | 0.11 glucose. |
| 2. By SHAFFER and HARTMANN method | ... | ... | ... | ... | ... | ... | ... | ... | 0.66 „ |
| Accepting the polarimeter figure for glucose, a solution was prepared containing 0.9 per cent. of glucose and 6 per cent. of borax. From this solution, mannitol in the original solution as estimated by the polarimeter | | | | | | | | | |
| | ... | ... | ... | ... | ... | ... | ... | ... | 1.917 mannitol. |
| Two portions of the original solution were treated with yeast and after alcohol extraction gave by polarimeter | | | | | | | | | |
| | ... | ... | ... | ... | ... | ... | ... | (1) | 1.926 mannitol. |
| | | | | | | | | (2) | 1.898 „ |
| These solutions showed zero rotations after the yeast treatment, showing that glucose was now absent. The SHAFFER and HARTMANN results bore this out, being 0.003 per cent. and 0.001 per cent. respectively, as glucose. | | | | | | | | | |
| TEST 2. | | | | | | | | | |
| Glucose present in the original solution— | | | | | | | | | |
| 1. By polarimeter | ... | ... | ... | ... | ... | ... | ... | ... | 0.11 glucose. |
| 2. By SHAFFER and HARTMANN method | ... | ... | ... | ... | ... | ... | ... | ... | 0.66 „ |
| A 0.9 per cent. glucose solution, prepared as in Test 1, gave by polarimeter | | | | | | | | | |
| | ... | ... | ... | ... | ... | ... | ... | ... | 1.912 mannitol. |
| A 0.5 per cent. glucose solution, prepared similarly, gave by polarimeter | | | | | | | | | |
| | ... | ... | ... | ... | ... | ... | ... | ... | 1.840 „ |
| Results obtained after yeast treatment and alcohol extraction of the total residue— | | | | | | | | | |
| Mannitol by polarimeter: (1) 1.84 per cent.; (2) 1.79 per cent. | | | | | | | | | |
| Mannitol by acetylation: (1) 1.81 per cent.; (2) 1.80 per cent. | | | | | | | | | |
| Rotations of the ether extracts without and with borax, zero in all cases. | | | | | | | | | |

Notes on Table XXII.

The polarimeter test for glucose on the original solution showed 0.11 per cent. The SHAFFER and HARTMANN test showed six times this amount. The polarimeter result was accepted for use in making up the solution into one containing 0.9 per cent. of glucose and 6 per cent. of borax, as required by the quicker method. The results obtained agreed very well with those given by the method of removing glucose by means of yeast. The difference between the glucose estimations by the different methods might have been due to the presence of laevo-rotatory substances or of optically inactive reducing compounds. If compounds were present which were laevo-rotatory and non-fermentable, these would have given an unmistakable negative reading after the yeast treatment. In the portions which were treated with yeast, zero polarimeter readings were obtained after the yeast treatment, while the results of the copper reduction tests were negligible, as shown in the table. It appears, therefore, that the higher results by the SHAFFER and HARTMANN method must have been due to optically inactive reducing compounds, which are formed from the glucose during the sterilization of the alkaline medium. This follows from the fact that while the glucose used in the experiment was a pure sample giving concordant analytical

figures by both the polarimeter and the SHAFFER and HARTMANN methods, the analysis of the solution after sterilization and before inoculation was :—

| Glucose— | Per cent. |
|---------------------------------------|-----------|
| (1) By polarimeter | 4.43 |
| (2) By SHAFFER and HARTMANN | 5.28 |

The results on the whole indicate that, provided that there are no interfering optically active bodies except glucose, either method will give satisfactory results.

The two methods described were now applied to further actual metabolism solutions, resulting from the growth of certain moulds on CZAPEK-Dox glucose solutions. The two moulds chosen for experiment were the white *Aspergilli* Ac. 55 and Ac. 56 which, from their carbon balance sheets, were known to give rise to large amounts of "carbon unaccounted for" and which, from previous qualitative experiments, were known to produce substantial amounts of mannitol from glucose (see Part IX). The experiments were undertaken with the double object of (1) testing the efficiency of the two methods on actual metabolism solutions, and (2) determining which of the two moulds gave the more satisfactory results from the point of view of mannitol production.

The results obtained are dealt with under each mould :—

A.—*Aspergillus* species, Ac. 56.

The solutions 14 M/A, 14 M/D, 14 M/L, 14 M/F, and 14 M/J were obtained in a set of comparative experiments to determine the effect of varying degrees of aeration on mannitol production by Ac. 56 when grown on CZAPEK-Dox glucose medium (see Part IX, p. 166). * Partial balance sheets were prepared for these solutions before the mannitol was estimated, and the figure obtained by deducting from the total carbon in solution that of the carbon present as glucose is given, after recalculation as gm. mannitol per 1,000 c.c., in column 5 under the heading "Equivalent of carbon in total metabolic products in solution."

Notes on Table XXIII.

The residue obtained on evaporation after the yeast and lead treatment was gummy and not easily extracted with alcohol. It will be seen in the last test that even the sixth extraction removed an appreciable amount of material. All extracts were examined without and with borax in the polarimeter and found to give negligible results so that practically no mannitol was removed by the extracting mixture. In all cases the polarimeter result on the extracted material is distinctly lower than its acetyl value, so that alcohol-ether treatment, while desirable from the point of view of extracting substances like glycerol which depress the polarimeter reading, does not give a product free from acetylisable compounds other than mannitol. Since interference in the polarimeter test has been studied and the result of interference in the above polarimeter

TABLE XXIII.—Analysis of solutions from *Aspergillus* species Ac. 56. Comparison of Results by Polarimeter and Acetylation Methods and the figure for total Metabolic Products in Solution. All results are expressed as gm. Mannitol per 1,000 c.c. solution.

| Solution. | Number of Alcohol-Ether Extractions. | Gm. Mannitol per 1,000 c.c. of Solution. | | | | | | |
|-----------|--------------------------------------|--|------------------|-----|-----|-----|---|--------|
| | | Polarimeter. | Acetylation. | | | | Equivalent of Carbon in Total Metabolic Products in Solution. | |
| 14 M/A | 1 | 1.167 | Solid Residue | ... | ... | ... | 2.325 | 3.405 |
| | | | Extract 1 | ... | ... | ... | 0.557 | |
| | | | Total | ... | ... | ... | 2.882 | |
| 14 M/D | 1 | 3.272 | Solid residue | ... | ... | ... | 5.132 | 7.865 |
| | | | Extract 1 | ... | ... | ... | 0.798 | |
| | | | Total | ... | ... | ... | 5.930 | |
| 14 M/L | 2 | 2.822 | Solid residue | ... | ... | ... | 4.678 | 9.622 |
| | | | Extracts 1 and 2 | ... | ... | ... | 1.900 | |
| | | | Total | ... | ... | ... | 6.578 | |
| 14 M/F | 3 | 3.664 | Solid residue | ... | ... | ... | 5.009 | 9.198 |
| | | | Extracts 1-3 | ... | ... | ... | 1.584 | |
| | | | Total | ... | ... | ... | 6.593 | |
| 14 M/J | 6 | 3.190 | Solid residue | ... | ... | ... | 4.018 | 13.032 |
| | | | Extracts 1-5 | ... | ... | ... | 1.532 | |
| | | | Extract 6 | ... | ... | ... | 0.265 | |
| | | | Total | ... | ... | ... | 5.815 | |

tests would not affect appreciably the results in Table XXIII, the polarimeter values are probably quite near the truth. Hence it may be broadly stated that of the total products of the metabolism of *Aspergillus* species Ac. 56 on glucose, only about a third is mannitol, while of the total acetylisable material only about half is mannitol. Thus considerable quantities of some polyhydroxyl compound must be present. This conclusion was verified by the identification of considerable quantities of glycerol in the metabolic products of *Aspergillus* species Ac. 56 (see Part IX, p. 167).

B.—*Aspergillus* species, Ac. 55.

The solution used was obtained from the growth of Ac. 55 on CZAPEK-DOX glucose solution under the usual conditions of restricted aeration (see Part IX). A number of

flasks were prepared and a full carbon balance sheet drawn up on a representative sample of the mixed solutions. The remainder contained a maximum of approximately 18.5 gm. mannitol if the assumption is made that mannitol is the only substance responsible for the "carbon unaccounted for" figure.

The solution was treated with yeast and lead acetate in the usual way and then evaporated *in vacuo* at a temperature not exceeding 60° C. The residue was a thick syrup which, after heating for some time on the water-bath at 60° C., became solid. This residue was dissolved in water, made up to 500 c.c. and the solution tested with the results shown in Table XXIV.

TABLE XXIV—Polarimeter and Acetylation Tests on a Metabolism Solution from *Aspergillus* species Ac. 55.

| Test No. | Conditions. | Gm. Mannitol in the Solution (500 c.c.). | |
|----------|---|--|---|
| 1 | 100 c.c. of the solution were extracted four times with alcohol. The polarimeter result is corrected by interpolation for the amount of glucose present. | Polarimeter. | Acetylation. |
| | | 16.00 | Solid residue ... 17.70 |
| | | | Extract No. 1 ... 0.89 |
| | | | Extracts 2-4 ... 1.18 |
| | | | Total ... 19.77 |
| 2 | 50 c.c. of the solution were extracted once. Polarimeter result corrected as in Test 1. | 15.54 | Rotation of extracts, without and with borax, negligible. |
| | | | Solid residue ... 18.58 |
| | | | Extract ... 0.70 |
| | | | Total ... 19.28 |
| | | | Rotation of extract without and with borax, negligible. |
| 3 | Two 50 c.c. quantities were evaporated and extracted once. The solid residues were made up into solutions containing 0.9 per cent. glucose and 6 per cent. of borax. Results were interpreted by means of Table VIII. | Green | Yellow |
| | | Light. | Light. |
| | | Test A ... 15.72 | 15.50 |
| 4 | The solution was tested by acetylation. In the titration, the end points were very difficult to determine on account of the dark colour of the solution. | Test B ... 15.78 | 15.66 |
| | | — | (i) 20.41 |
| | | — | (ii) 19.44 |
| 5 | The mannitol in the solution was estimated by polarimeter. The result was corrected by interpolation for the amount of glucose present. | 15.71 | — |

Notes on Table XXIV.

1. The polarimeter results agree quite well among themselves and indicate that concordant results may be expected in practice from the two methods.

2. The average of 15.7 gm. of mannitol from the polarimeter figures is 85 per cent. of the "carbon unaccounted for" (equivalent to 18.5 gm. mannitol). Hence *Aspergillus* species Ac. 55 is much to be preferred to *Aspergillus* species Ac. 56 for mannitol production since the corresponding figure for the latter mould is less than 50 per cent.

3. It will be seen that the figures obtained by the acetylation method (average 19.74) are considerably higher than those given either by the polarimeter method (average 15.7) or by the carbon balance sheet (18.5). This is to be explained by the fact that the solution was fermented by yeast after the carbon balance sheet had been prepared but before the polarimeter and acetylation estimations. Hence the glycerol formed during the fermentation of the residual glucose by yeast, which would amount to from 0.5 to 1 gm. in weight, is not included in the figure 18.5 obtained from the carbon balance sheet. Since glycerol is not estimated by the polarimeter it will not be included in the polarimeter figure (15.7), so that the acetylation figure should in any case be from 0.5-1.0 higher than either the polarimeter or carbon balance sheet figures.

4. It is evident from the reasonable agreement between the figures obtained by the different methods that almost all the "carbon unaccounted for" is present as mannitol, so that *Aspergillus* species Ac. 55 should be a satisfactory mould for mannitol production since the product obtained will be in a reasonably pure condition and hence easier to isolate.

Discussion of Results and Description of Method.

A method was sought for estimating mannitol produced in the fermentation of glucose by moulds. The chief interfering substance was glucose.

Precipitation methods involving the use of benzaldehyde and *p*-nitrobenzoyl chloride were tried, but were found to give low and irregular results. Acetylation of pure mannitol gave results from 99 per cent. to 100 per cent. on the pure compound. The details of the method were almost the same as those used in the analysis of glycerol.

The property possessed by mannitol of becoming optically active in borax solution was also investigated. The best concentration of borax in the solution was found to be 6.00 gm. $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$ per 100 c.c. This concentration gave quite large readings in the polarimeter while greater concentrations gave trouble from crystallisation. A series of tests was carried out with 6 per cent. of borax and 0.1 per cent.-5.0 per cent. of mannitol. A curve was drawn from these results, and from the smoothed curve a table was drawn up giving gm. mannitol corresponding to rotations from 0.1° to 4.5° . Mercury yellow and green lights were both used.

Glycerol being a product of the fermentation of glucose by yeast, the effect of glycerol

on the mannitol rotation was investigated. Results are given in Table III. The mannitol rotation is depressed by amounts depending on the concentration of the glycerol.

The effect of glucose on mannitol rotation in borax solution was also investigated. The behaviour of glucose itself in 6 per cent. borax solution was first tested. At concentrations below 0.9 per cent. of glucose, the rotation was slightly but definitely negative. Above 0.9 per cent., at which point the solutions are optically inactive, the curve became positive and slowly approached the straight line found when glucose is tested polarimetrically in pure water solution. As very little reference could be found to this laevo-rotation in the literature, this point was tested thoroughly and was found to hold good with the double compound of glucose and sodium chloride which is easily prepared in a pure condition.

The method of estimating mannitol in presence of glucose by removing the latter with yeast, was tested on synthetic mixtures of mannitol and glucose in various proportions. Results given in Table XII show 98-99 per cent. by polarimeter and 105 per cent. by acetylation. In the next series of tests an attempt was made to extract the glycerol from the test quantity by means of alcohol before acetylation. The polarimeter results, uncorrected for glycerol and glucose were 97-100 per cent. and after correction 98-100 per cent., but the extraction and acetylation results were erratic. Much better results were obtained by evaporating the whole of the sample to dryness and repeatedly extracting the residue, but even with this method the acetyl results were sometimes high. This extraction test is useful, however, as a product after evaporation may appear to be solid and yet contain a large percentage of liquid material. This was found with unknown mixtures (Table XXIII) where half of the product after evaporation was found to be mannitol and the remainder a stiff syrup soluble in alcohol-ether. Trouble was occasionally experienced with the solutions used in the polarimetric determinations due, probably, to too high a concentration of material. The point was not definitely proved, but the trouble did not appear when the concentration was kept below a certain value. The effect of salt concentration was found to be small.

For control purposes, an attempt was made to estimate mannitol in presence of glucose. Two curves were prepared and tables deduced from them showing concentration of mannitol corresponding to rotation in 6 per cent. borax solution containing (1) 0.9 per cent. glucose, and (2) 0.5 per cent. glucose. In practice, the glucose content was first estimated polarimetrically and then a solution was prepared containing 0.9 per cent. or 0.5 per cent. of glucose either by dilution of the test solution or, if necessary, by addition of glucose. This method was tested on fermentation solutions and found to give quite good results against the method employing yeast treatment. The removal of the yeast treatment from the test quickens control considerably. The presence of unfermentable optically active bodies would of course interfere with both methods.

Details of the methods finally developed are as follows :—

The Estimation of Mannitol in Fermentation Solutions.

1. Method involving the Removal of Residual Glucose by Yeast.

A suitable quantity of the fermentation solution is measured into a litre conical flask and sterilized. The residual glucose in the solution is then removed by fermentation with a pure culture of *Saccharomyces cerevisiae*. The fermentation process requires about three days. The contents of the flask are then neutralised with N/1 alkali. The indicator used is bromthymol blue, and is used externally in the same way as litmus paper. 10 c.c. of 20 per cent. normal lead acetate solution are added. The flask is allowed to stand until the precipitate of lead salts which is formed has settled. This requires one to two hours. The solution is filtered on a Buchner funnel through a No. 40 Whatman filter paper and a layer of calcined kieselguhr. The filtrate is transferred to a conical flask and again made neutral to bromthymol blue. 10 c.c. of 10 per cent. basic lead acetate solution are added. A precipitate forms which requires to stand overnight to settle. The contents of the flask are then filtered as before. The filtrate is transferred to a conical flask and is treated with hydrogen sulphide. After the precipitate has settled, the contents of the flask are filtered as before. The filtrate is evaporated under reduced pressure to small bulk in a large Claisen flask which is placed in a water bath, the temperature of which is kept just under 60° C. The solution is then transferred to a small Claisen flask and evaporated to dryness. When no further distillate is coming over, 20 c.c. of absolute alcohol are introduced into the flask and distilled off. This alcohol treatment is repeated once. 40 c.c. of absolute alcohol are then introduced into the flask, which thereafter is attached to a dry reflux condenser fitted with a calcium chloride tube. The alcohol is boiled on a hot water bath for one hour. The flask is removed and stoppered and allowed to cool, the stopper being fitted with a calcium chloride tube. After the flask is cool 160 c.c. of sodium-dried ether are added. The flask is shaken, and after the contents have settled again, the alcohol-ether solution is filtered off through a filter paper into a dry beaker. The residue in the flask and the filter paper are washed with 50 c.c. of 1 : 4 alcohol-ether mixture, used in small quantities. The filter paper is kept. The filtrate is evaporated to dryness and the residue kept for further examination. The solvent remaining in the Claisen flask is removed under reduced pressure. The extraction process is repeated twice, a fresh filter paper and beaker being used each time. The filter papers are washed on the funnel with hot water, the washings being added to the small Claisen flask. The residual solvent in the flask is taken off under reduced pressure at a temperature not greater than 60° C. The contents of the flask are then made up to standard volume, usually 250 c.c. The extracts are made up to 100 c.c., combined or treated separately as desired. The main solution containing the mannitol residue is tested as follows :—

1. *Glucose*.—The solution is tested polarimetrically without further treatment. If possible a 4 dm. tube is used, but it is occasionally necessary to use a shorter tube

when the mercury light fails to penetrate the 4 dm. column of liquid. Zero rotations show that the glucose has been destroyed.

2. *Mannitol by Polarimeter.*—50 c.c. or other suitable quantity of the solution is made neutral to bromthymol blue and, with 6.00 gm. of borax ($\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$), made up to 100 c.c. The borax solution is tested in the polarimeter. The reading is interpreted by means of Table II in this paper, and hence the weight of mannitol in the standard solution is calculated.

3. *Acetyl Value.*—50 c.c. or other suitable quantity of the solution is evaporated to dryness in a 250 c.c. round-bottomed flask with a ground neck. This is carried out by immersing the flask in a bath of boiling water and passing hot air through a glass tube on to the surface of the liquid. The air is heated by means of a coil also immersed in a bath of boiling water. The residue in the flask is treated with 3 gm. of fused sodium acetate and 7.5 c.c. of acetic anhydride, the flask being heated on a sand-bath and the acetic anhydride kept just boiling. After an hour, the flask is lifted clear of the sand-bath and allowed to cool slightly. 50 c.c. of water at 80°C . are added through the condenser and, without removing the condenser, the flask is placed in a boiling water bath. After half an hour, the contents of the flask are transferred to a litre conical flask with hot water. The solution is allowed to cool. The solid hexa-acetate is broken up with a glass rod. The solution is made neutral to phenolphthalein. 50 c.c. of N/1 standard alkali are added to the flask which is then fitted to a reflux condenser. The contents of the flask are boiled gently for 20 minutes and then the flask is cooled and the excess alkali is titrated. From the figures obtained, the amount of mannitol in the quantity of solution taken is calculated.

Analysis of the Alcohol-Ether Extracts.—The extracts are made up together or separately to 100 c.c. This standard solution is tested in the same way as described above for the main solution, except that the following quantities are used :—

1. *Rotation with borax.*—50 c.c. are evaporated to about 25 c.c., treated with 3 gm. of borax and made up to 50 c.c.
2. *Acetylation.*—20 c.c. of the solution are used with 1 gm. of fused sodium acetate and 3 c.c. of acetic anhydride. In the hydrolysis of the acetyl compound, 25 c.c. N/10 alkali are used.

Note on Infection.—The standard solutions of mannitol residue and the alcohol-ether extracts are liable to become infected with consequent loss of mannitol if precautions are not taken. It is advisable to measure out the required quantities as soon as possible after the standard solutions have been prepared and to carry out the tests at once. Borax solutions are not liable to infection. In the acetylation estimation, if a test quantity is measured out and cannot be evaporated within a day, it may be preserved by adding a few drops of toluene.

2. *Control Method, not involving Removal of Residual Glucose by Yeast.*

A suitable quantity of the fermentation solution is measured into a conical flask, and made neutral to bromthymol blue. Thereafter the solution is treated with lead exactly as described in Method 1. Excess lead is removed with hydrogen sulphide. After filtration the solution is evaporated in a Claisen flask as described above, and finally made up to standard volume. The standard solution is tested in the polarimeter, and the result calculated to percentage of glucose in the solution. If this figure is greater than 0.9 per cent., the quantity containing 0.45 gm. glucose is calculated. This quantity, measured out from a burette, with 3.00 gm. borax added, is made up to 50 c.c. This solution is tested in the polarimeter and the results interpreted by means of Table VIII, which gives the relation between rotation and gm. of mannitol present in a solution containing 0.9 per cent. of glucose. If the solution contains less than 0.9 per cent. of glucose, 50 c.c. are taken and evaporated to, say, 25 c.c. The calculated quantity of a day-old 5 per cent. glucose solution, equivalent to the difference between 0.45 gm. glucose and the amount in 50 c.c. of the standard solution is added along with 3.00 gm. of borax. The solution thus prepared is made up to 50 c.c. in a standard flask. This solution is tested in the polarimeter as before.

If required, or if conditions are more suitable, the glucose concentration in the polarimeter solution may be made up to 0.5 per cent. and Table X used to interpret the results.

Summary.

Two methods are described for the estimation of mannitol, in the presence of residual glucose, in fermentation solutions. Both methods are based on the optical rotation of mannitol in borax solution. In one method the residual glucose is removed by fermentation by *Saccharomyces cerevisiae*. The other method is based on the observation, believed to be new, that glucose at a concentration of 0.9 per cent. in a 6 per cent. solution of borax ($\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$) is optically inactive, whereas at concentrations below 0.9 per cent. it is actually laevo-rotatory.

Studies in the Biochemistry of Micro-organisms.

PART XI.—*On citromycetin, a new yellow colouring matter produced from glucose by species of Citromyces.*

By ARTHUR CLEMENT HETHERINGTON and HAROLD RAISTRICK.

The genus *Citromyces* WEHMER comprises a number of species which were considered by WEHMER to be unique in the fact that they produce considerable quantities of citric acid by the fermentation of sugar. Because of this, WEHMER gave to the genus the name of *Citromyces*. The genus is closely related to *Penicillium* and *Aspergillus*; and has in fact been dropped by THOM in his recent book on the genus *Penicillium* (1929). THOM prefers to regard all species previously regarded as *Citromyces*, as species of *Penicillium* (see Part IV).

Physiologically the genus *Citromyces* was considered to be distinguished from other genera by the production of citric acid, but more recent work has shown that not only is the production of citric acid a fairly common characteristic of other genera, particularly of *Aspergillus* and *Penicillium*, but that, quantitatively, certain species which are not *Citromyces* give much larger yields of citric acid.

During the work of preparation of carbon balance sheets for a number of species of moulds, balance sheets were prepared for a few species of *Citromyces* and seemed to have a certain amount of interest (see Part IV). The history of the species used in the work described in this paper is as follows :—

1. *Citromyces* sp. Catalogue No. Ad. 6. Obtained from the National Collection of Type Cultures, Lister Institute, via Mr. F. T. BROOKS, Cambridge.
2. *Citromyces* sp. Catalogue No. Ad. 7. Isolated at Ardeer.
3. *Citromyces* B. WEHMER, Catalogue No. Ad. 67. Purchased from the National Collection of Type Cultures, Lister Institute, No. 606.
4. *Citromyces glaber* WEHMER. Catalogue No. Ad. 68. Purchased from the Centraalbureau voor Schimmelcultures at Baarn.
5. *Citromyces Pfefferianus* WEHMER. Catalogue No. Ad. 69, also purchased from Baarn.

In view of the fact that an interesting and new biochemical product was obtained from all the above five species we considered it desirable to obtain expert opinion as to the authenticity of these cultures. For this reason we sent sub-cultures to Professor CARL WEHMER, of the Technical High School, Hanover, the originator of the genus *Citromyces*, and to Professor PHILLIP BOURGE, of the University, Louvain.

Professor WEHMER, to whom we sent each of the above cultures, together with three other cultures of *Citromyces*, i.e., Catalogue Nos. Ad. 21, Ad. 71 and Ad. 73, wrote :

“ Ihre *Citromyces*-Formen sind hier microscopiert, auch auf Zuckerlösung mit Mineralsalzen, einige ebenfalls auf Gelatine, Agar und Kartoffeln cultiviert.

“ Das Resultat ist wenig ermutigend, die Masse für Sporen, Köpfchen, Sporenträger mit Sterigmen sind nahezu oder ganz übereinstimmend, sodass ich mich nicht getraue, den einzelnen Formen bestimmte Speciesnamen zu geben. Es mag sein, dass man bei detailliertem Studium gewisse feine morphologische Unterschiede feststellen kann, dazu bedarf es aber langer Arbeit. In wie weit sich culturelle und chemisch-physiologische Differenzen verwerten lassen, muss ich dahingestellt lassen, ob sie zu einer Species-Trennung in dieser Gattung berechtigen, wäre eine weitere Frage. Man müsste auch das Verhalten bei höherer Temperatur, bei Luftmangel, etc., feststellen bez. kennen. Beim Wachstum auf Zuckerlösung ergeben sich 2 Gruppen, die einen verfärben diese auch nach längerer Zeit kaum (7, 21, 68 u.a.), die anderen bilden gelbliches *Pigment*; Deckenunterseite jener bleibt farblos. Die Säurebildung kann bei ein und derselben Form erheblich variiren, sodass bei Kreidegegenwart auf 15 Prozentiger Saccharose-Lösung mit Nährsalzen bald wenig, bald reichlich Calciumcitrat erscheint.

“ Ich bin der Meinung, dass die geschickten 8 Culturen mehrfach identisch sind und sich vielleicht auf 2 verschiedene Formen oder Arten verteilen lassen; natürlich ist das nur eine *Schätzung* auf Grund des von mir Gesehenen, sie bedürfte genauerer Prüfung.

“ Im übrigen handelt es sich bei allen 8 um notorische *Citromyces*-Formen, auch Ad. 7 und Ad. 21 sind solche; um diese hatten Sie speciell angefragt.”

Professor BOURGE, to whom we sent each of the above five cultures together with *Citromyces* cultures Ad. 71 and Ad. 73 wrote :—“ Ad. 7 est identique à l'espèce reçue de Prof. Pollacci, Istituto di Botanica della R. Università di Pavia; cette année même sous l'étiquette *Citromyces Pfefferianus*.

“ Votre *Citromyces Pfefferianus* Ad. 73 est identique à mon *Citromyces Pfefferianus*, No. 162, reçu de Pribram en 1911, très peu après la mort de Kral qui le tenait de Wehmer.

“ Le *Citromyces* B. WEHMER Ad. 67 est probablement le véritable. Il se caractérise par la formation de grains bruns insolubles. La saveur du liquide est citrique pure, pas amère comme celle de Ad. 6.

“ *Citromyces glaber* Ad. 68 fait très peu d'acide, mais le liquide n'est pas amer. L'exactitude de l'étiquette est simplement possible.

“ Ad. 71 *Citromyces lacticus* MAZÉ et PERRIER est très rapproché de *Citromyces Pfefferianus* (Kral-Pribram). Il est moins actif. Le revers de thalle sur liquide sucré est d'un

orangé plus marqué dans les replis. Ce sont des différences de détail, paraissant négligeables.

“ En résumé :—

Ad. 6 = Voisin de *Citromyces Pfefferianus* de Pavie.

Ad. 7 = *Citromyces Pfefferianus* de Pavie non de Wehmer.

Ad. 67 *Citromyces* B. WEHMER = probably genuine.

Ad. 68 *Citromyces glaber* WEHMER = *Citromyces glaber* WEHMER probably.

Ad. 69 *Citromyces Pfefferianus* Baarn = *Citromyces Pfefferianus* de Pavie pas de Wehmer.

Ad. 71 *Citromyces lacticus* = *Citromyces Pfefferianus* WEHMER (?).

Ad. 73 = *Citromyces Pfefferianus* WEHMER !

“ Votre citromycétine est donc tirée de tous *Citromyces*. Soyez en paix sur ce point.”

We wish to express our indebtedness to both Professor WEHMER and Professor BOURGE for their kindness in examining our cultures.

The salient features of the balance sheets of the five species Ad. 6, Ad. 7, Ad. 67, Ad. 68 and Ad. 69, which are given in detail in Part IV, are the following :—

TABLE I.—Principal data in carbon balance sheets of five species of *Citromyces*.

| Species. | Catalogue No. | Experiment No. | Carbon unaccounted for. | Carbon in non-volatile acids. | Carbon in Volatile Neutral Compounds. | Carbon in Synthetic Compounds. | Increase in Acidity, c.c. N/1 per 250 c.c. |
|------------------------------------|---------------|----------------|-------------------------|-------------------------------|---------------------------------------|--------------------------------|--|
| | | | gm. | gm. | gm. | gm. | c.c. |
| <i>Citromyces</i> sp. ... | Ad. 6 | 134 | 0.620 | 0.345 | 0.126 | 0.191 | 8.4 |
| <i>Citromyces</i> sp. ... | Ad. 7 | F 5 | 0.566 | 0.402 | 0.101 | 0.218 | 6.6 |
| <i>Citromyces</i> B. ... | Ad. 67 | F 74 | 0.686 | 0.304 | 0.208 | 0.036 | 4.1 |
| <i>Citromyces glaber</i> WEHMER | Ad. 68 | F 75 | 0.393 | 0.341 | 0.059 | 0.093 | 7.6 |
| <i>Citromyces Pfefferianus</i> | Ad. 69 | F 76 | 0.567 | 0.315 | 0.071 | 0.090 | 7.9 |

NOTE.—The values given for the different classes of carbon in columns 4, 5, 6 and 7 are expressed in the same manner as in the carbon balance sheets given in Part IV, *i.e.*, in gm. of carbon per 250 c.c. of medium.

Consideration of the balance sheets shows that all these species produce considerable amounts of acid and of “carbon unaccounted for,” and in two cases, at any rate, of “synthetic carbon.” They are distinguished biochemically, however, by the very remarkable reaction that culture solutions, on which any of these species have grown, give with ferric chloride solution. On the addition of a few drops of ferric chloride to the culture fluid from a mature culture on CZAPEK-Dox 5 per cent. glucose solution, a very intense greenish-black coloration is obtained, and provided that the culture has been sufficiently

active, there is a copious deposition, on standing, of a dark brown, amorphous, iron precipitate. This reaction is so intense that solutions from an active culture will still give a very strong reaction even after diluting to 100 times the original volume.

The work described in this paper deals with the isolation of the fermentation product responsible for this coloration and with its chemical investigation. This product has been isolated in the form of a yellow crystalline compound to which it is proposed to give the name "citromycetin," and it may be said safely that this product is more typical of, and specific for, the genus *Citromyces* than is citric acid, since, of several hundred cultures of different species from different genera, only a few species of *Citromyces* produce citromycetin, or give the colour reaction which is typical of it. The colour reaction is not given by all species of *Citromyces*, and it varies in intensity in those species that give a positive reaction.

Influence of different factors on the yield of citromycetin.

No prolonged effort has been made to define optimum conditions for the production of citromycetin. It is important, therefore, to note that the yields given are not by any means as large as are probably obtainable.

From time to time, however, various isolated observations have been made, and these, while necessarily incomplete, are given as indications of the lines on which work might be carried out if it were desired to increase the yield of material.

(a) *Influence of aeration.*—A copious supply of air is necessary for the production of citromycetin. It was first noticed in the metabolism experiments, where aeration was strictly controlled and was very limited, that the colour reaction with ferric chloride, of any of the *Citromyces* examined, was very much less intense than with the same culture grown in flasks plugged with cotton wool. Analysis of the air contained in the respective flasks showed that, while in the metabolism flasks practically all the oxygen had disappeared on standing overnight, those flasks which were plugged with cotton wool had usually a copious supply of oxygen.

The effect of aeration was also shown in a very marked manner in some of the earlier large scale experiments. Erratic results were often obtained, and in the light of later experience it seems probable that these were due to unequal aeration of the twelve trays since, because of the method of aeration adopted, it was impossible to ensure uniform aeration of all the trays. Thus it was noticed several times that, of the twelve trays in the same tank (sterilizer-incubator), all of which contained equal amounts of culture medium and had been sown with equal amounts of the same culture, some gave satisfactory yields of citromycetin while others gave scarcely any. (See Table V.)

(b) *Effect of concentration of iron salts in the medium.*—The CZAPEK-DOX medium used throughout all the metabolism experiments contained 0.001 per cent. of ferrous sulphate. Comparative experiments with media identical in all respects except that they contained varying amounts of ferrous sulphate showed that the yield of citromycetin,

judged by its colour reaction with ferric chloride, increases with the amount of ferrous sulphate in the medium up to a maximum of 0.02 per cent. and after that decreases with higher concentrations of ferrous sulphate.

(c) *Influence of the nature of the containing vessel.*—While it is evident that the erratic results obtained with the different trays in one and the same tank were probably largely caused by inequality of aeration, it was felt that this was not the only explanation. The trays in which the mould was grown in the tank were made of iron covered with enamel. After being in use for some time these trays showed signs of pitting. The influence of the nature of the trays on the yield of citromycetin was shown conclusively by comparing the yields obtained in these enamelled trays with those given in fused quartz trays of the same size obtained from the Thermal Syndicate, Ltd. ("Vitreosil"). The results are summarized in Table V under tanks 11 and 12. In these experiments six enamelled trays were used and six silica trays. These were all enclosed in the same tank and every effort was made to keep all other conditions constant. Not only was the yield of citromycetin uniformly larger in the silica trays than in the iron trays but the quality of product obtained was very much superior.

(d) *Influence of temperature of incubation and source of carbon.*—In all the large scale experiments the tank was placed in a room the temperature of which fluctuated very considerably. The results of experiments about to be described show that this would have a marked effect on the yield of citromycetin obtained.

Five litres of medium were made up of the same composition as that described on p. 215 with the exception that no glucose was added to it at this stage. The medium was divided into two halves and to one half was added 125 gm. of pure glucose (5 per cent.) while to the other half was added 125 gm. of Price's pure glycerol. These two media were now tubed in 10 c.c. quantities and sown with a spore suspension of *Citromyces* sp. Ad. 7. Forty tubes of each medium were then incubated at a series of different temperatures. These temperatures which, with the exception of room temperature, fluctuated very little, were taken three times per day during the course of the experiment, and the following are the averages of these figures:—

- (1) Room temperature = 14.7°C ; (2) 23.5°C ; (3) 27.5°C ; (4) 29.9°C ; (5) 31.8°C ;
(6) 36.9°C .

The average temperature of the room in which the tank was incubated over the same period was 23.8°C ., but this temperature fluctuated over a much wider range than any of the temperatures given above.

Four tubes of each medium, at each temperature, were examined at suitable intervals and the amount of citromycetin produced was estimated by the method given on p. 232. This method was applied to the glucose set of tubes exactly as is given on p. 232. With the glycerol set of tubes all that was found necessary was an iodine estimation, since alkaline iodine was shown to have no appreciable oxidising effect on pure glycerol. The results are summarized in the following tables.

TABLE II.—Production of citromycetin from a glycerol medium at different temperatures.

| Average Temperature of Incubation. | Iodine Absorption Figure in c.c. of N/10 Iodine per 100 c.c. Medium after Number of Days' Incubation indicated at Head of each Column. | | | | | | | | | |
|------------------------------------|--|---------|----------|----------|----------|----------|----------|----------|----------|----------|
| | 6 days. | 8 days. | 12 days. | 14 days. | 16 days. | 20 days. | 23 days. | 26 days. | 30 days. | 35 days. |
| Room temp. | 25.5 | 33.3 | 72.0 | 100.3 | 154.8 | 199.3 | 248.0 | 273.0 | 275.5 | 286.3 |
| 23.5° C. | 26.3 | 51.5 | 128.8 | 152.3 | 175.5 | 207.3 | 208.3 | 204.5 | 194.0 | 207.3 |
| 27.5° C. | 29.8 | 47.3 | 96.3 | 96.8 | 103.0 | 112.8 | 120.0 | 119.8 | 108.5 | 107.3 |
| 29.9° C. | 32.3 | 55.5 | 89.8 | 96.5 | 116.5 | 124.3 | 119.3 | 114.0 | 115.3 | 124.5 |
| 31.8° C. | 27.5 | 38.3 | 84.8 | 73.3 | 77.8 | 89.3 | 71.3 | 66.8 | 72.0 | 82.5 |
| 36.9° C. | — | 39.8 | 33.0 | 36.3 | 37.0 | 36.8 | 37.3 | 38.5 | 34.3 | 48.8 |

TABLE III.—Production of citromycetin on glucose medium at different temperatures.

| Average Temperature of Incubation. | Iodine Absorption Figure in c.c. of N/10 Iodine per 100 c.c. Medium after Number of Days' Incubation indicated at Head of each Column. | | | | | | | |
|------------------------------------|--|----------|----------|----------|----------|----------|----------|---------------------------------------|
| | 8 days. | 12 days. | 14 days. | 16 days. | 20 days. | 23 days. | 28 days. | 35 days. |
| Room temp. | 26.1 | 51.9 | 53.7 | 55.1 | 46.2 | 63.9 | 78.6 | 87.7 |
| 23.5° C. | 35.1 | 59.7 | 51.1 | 61.7 | 84.1 | 108.1 | 119.7 | 104.8 |
| 27.5° C. | 23.6 | 62.1 | 57.8 | 86.0 | 88.4 | 101.0 | 89.2 | } No material available for analysis. |
| 29.9° C. | 44.2 | 70.1 | 77.3 | 86.9 | 106.1 | 105.2 | 96.4 | |
| 31.8° C. | 8.8 | 28.6 | 27.7 | 28.8 | 37.0 | 33.4 | 24.6 | |
| 36.9° C. | Nil | Nil | Nil | Nil | Nil | Nil | Nil | |

These results indicate that not only is the production of citromycetin influenced very considerably by the temperature of incubation but that the optimum temperature is very different on a glycerol medium from that on a glucose medium. Thus with a glycerol medium the yield of citromycetin is at a maximum when the mould is cultivated at about 14.7° C. As the temperature increases the yield of citromycetin steadily falls. On the other hand, with a glucose medium the optimum temperature seems to lie between 23.5° C. and 29.9° C., and the yield decreases on either side of this range. It also appears that better yields of citromycetin may be expected from a 5 per cent. glycerol medium than from a 5 per cent. glucose medium. Hence in the later large scale experiments 5 per cent. glycerol was used as the medium.

Ferric chloride tests and acidities determined by titration to phenolphthalein were carried out at the same time as the estimations of citromycetin, and it was found that the three different estimations invariably ran parallel, *i.e.*, the larger the amount

of citromycetin the more intense the colour reaction and the stronger the acidity of the medium.

(e) *Influence of concentration of glucose on production of citromycetin.*—A qualitative experiment in test tubes had been carried out on the growth of different species of *Citromyces* on varying concentrations of glucose in CZAPEK-Dox solution. At the end of the experiment, after 42 days' incubation, a few of these tubes were available and estimations of citromycetin were carried out. The results given in Table IV were mostly obtained on two tubes of medium (10 c.c.) and in some cases only on one, so that they can only be accepted in a general sense.

TABLE IV.—Influence of concentration of glucose on production of citromycetin.

| Culture. | 5 per cent. Glucose. | | 10 per cent. Glucose. | | 20 per cent. Glucose. | | 30 per cent. Glucose. | |
|----------|-----------------------------------|--|-----------------------------------|--|-----------------------------------|----------------|-----------------------------------|--|
| | Residual Glucose by Polari-meter. | Citro-mycetin in c.c. N/10 Iodine per 100 c.c. Medium. | Residual Glucose by Polari-meter. | Citro-mycetin in c.c. N/10 Iodine per 100 c.c. Medium. | Residual Glucose by Polari-meter. | Iodine Figure. | Residual Glucose by Polari-meter. | Citro-mycetin in c.c. N/10 Iodine per 100 c.c. Medium. |
| Ad. 6 | per cent. | c.c. | per cent. | c.c. | per cent. | None available | per cent. | c.c. |
| Ad. 6 | 0·07 | 143·2 | 1·66 | 358·0 | 13·52 | | 24·0 | 318 |
| Ad. 7 | 0·47 | 125·1 | 1·28 | 329·9 | 14·59 | | 26·1 | 191 |
| Ad. 67 | 0·08 | 155·8 | 0·63 | 295·4 | 13·99 | | 26·4 | 219 |
| Ad. 68 | 0·18 | 56·7 | 4·09 | 167·9 | 19·21 | | — | — |
| X. 92 | 0·12 | 142·7 | 0·14 | 437·0 | — | | 22·7 | 152 |
| X. 103 | Nil | 158·2 | 0·48 | 446·9 | 13·78 | | 25·6 | 310 |

These results indicate that larger yields of citromycetin may be expected if a more concentrated solution of glucose is used than the customary 5 per cent. Indeed the figures given for X. 103 on 10 per cent. glucose indicate that under these conditions a yield of approximately 20 per cent. of citromycetin has been obtained.

Preparation of citromycetin.

For the preparation of citromycetin in quantity the combined incubator-sterilizer described in Part VII was used.

Sixty litres of a modified CZAPEK-Dox medium, containing twenty times the usual amount of iron, were made up of the following composition :—

| | | | | | | | |
|---------------------------------------|----|----|----|----|----|----|------------|
| NaNO ₃ | .. | .. | .. | .. | .. | .. | 2.0 gm. |
| KH ₂ PO ₄ | .. | .. | .. | .. | .. | .. | 1.0 gm. |
| KCl | .. | .. | .. | .. | .. | .. | 0.5 gm. |
| MgSO ₄ . 7H ₂ O | .. | .. | .. | .. | .. | .. | 0.5 gm. |
| FeSO ₄ . 7H ₂ O | .. | .. | .. | .. | .. | .. | 0.2 gm. |
| Glucose (or glycerol) | .. | .. | .. | .. | .. | .. | 50 gm. |
| Water | .. | .. | .. | .. | .. | .. | 1,000 c.c. |

This medium was made by dissolving all the constituents in the water, except the potassium phosphate, which was dissolved in a separate small portion of water and finally mixed with the main bulk. Five litres of this medium were distributed in each of the twelve incubating trays of the combined incubator-sterilizer, and during distribution, the liquid was kept stirred by a current of air in order to ensure uniform mixing of the precipitated phosphate. The apparatus was then closed and sterilized by steaming for an hour on each of three consecutive days. After cooling, each tray was sown with a suspension of spores from a beer wort agar Roux bottle culture of *Citromyces* sp. Ad. 7, which was found most suitable for the purpose. The spore suspension was made in sterile distilled water and introduced by means of a sterile pipette through the inoculation openings of the tank. The spores were then distributed over the surface of the trays by means of a sterile bent aluminium rod. After sowing, the mould was not further disturbed, and was incubated at an average temperature of 22°–24° C. The mould grew very quickly, and in successful experiments a dark green, heavily sporing, felt was established on all trays. During the course of incubation a continuous stream of sterile air was passed over the surface of each tray as it had been found to be essential in order to ensure good yields of material that the mould should be thoroughly aerated. From time to time samples were withdrawn and a measure of the quantity of citromycetin was obtained by the method given on p. 232. This figure slowly increased until it reached a maximum when practically all the glucose had disappeared, which usually happened in from 10 to 12 days.

At the end of this time the culture solution was filtered from the mycelium which was then thoroughly extracted by grinding with small amounts of water. The filtrate and filtered washings were then acidified with sulphuric acid (50 per cent. by volume), 100 c.c. of this concentration being added with vigorous stirring to the contents of one tank. On acidification, the culture solution, which was initially dark brown in colour, immediately became lighter and deposited a considerable quantity of a brownish black amorphous precipitate, the nature of which has not been investigated except to prove that it contains practically no citromycetin. This precipitate was filtered off and the clear orange yellow filtrate evaporated *in vacuo* at a low temperature. During the evaporation crude citromycetin separated in yellowish brown crystalline crusts. The whole solution was evaporated to about 3 to 5 litres and allowed to stand. Almost all the citromycetin separated out on standing and was filtered off, carefully washed and dried. This constituted the crude citromycetin. The mother liquors contain considerable quantities of citric acid which may be isolated as a calcium salt after removal of the sulphuric acid.

The results obtained in twelve tanks are summarized in Table V.

Purification of crude citromycetin.

The crude product contains, in addition to citromycetin, varying amounts of a brownish black tarry material which interferes considerably with the crystallisation.

TABLE V.—Details of “Tank” experiments for preparation of Citromycetin. Organism used—*Citromyces* sp. Ad. 7.

| Tank. | Carbon Compound Fermented. | Period of Incubation. | | | Location of Tank. | Type of Tray used. | Residual Glucose by Polarimeter. | Estimated Citromycetin as c.c. N/10 Iodine per 100 c.c. Medium. | Yield of Crude Citromycetin. | Remarks. |
|-------|--|-----------------------|---|----------------------------|--|---|---------------------------------------|---|---|---|
| | | Start. | Finish. | Duration in Days. | | | | | | |
| 1 | Glucose | 21.12.25 | Tray 6 taken off 11.1.26 Remainder 18.1.26 | 21 28 | Tank incubated in sterilizing room of old Biochemical Lab. | Enamelled iron | Per cent. 0.13 | — | 3.8 gm. | Tray 6 was the only tray of the whole 12 giving a reasonable ferric chloride reaction, and it was from the contents of this tray that the 3.8 gm. were obtained. None of the other trays gave a good reaction with ferric chloride and no citromycetin could be isolated from any of them. The temperature of the sterilizing room in which Tanks 1–10 were incubated fluctuated very widely ranging between 10°–26° C. in the earlier tanks and 20°–26° C. in the later tanks where an attempt was made to lessen the fluctuations. |
| 2 | Glucose in 9 trays. Maltose in 3 trays. | 29.1.26 | 3 trays on 9.2.26 5 trays on 10.2.26 4 trays on 11.2.26 | 11 12 13 | Ditto | Ditto | 0.2–0.6 | — | 125.7 gm. from 9 glucose trays. 50.5 gm. from 3 maltose trays. | The yield of citromycetin judged from the ferric chloride reaction varied considerably from tray to tray and although a considerable amount of crude material was isolated subsequent investigations showed that the content of citromycetin was not very large. No advantage was evident in using maltose as compared with glucose. |
| 3 | Glucose | 22.4.26 | 4 trays on 3.5.26 5 trays on 4.5.26 3 trays on 5.5.26 | 11 12 13 | Ditto | Ditto | 0.14–0.58 | — | 116.4 gm. | In this tank 0.02 per cent. of ferrous sulphate was used in the medium for the first time, Tanks 1 and 2 having only contained 0.001 per cent. It was noticed that the mycelium in all trays was covered with spore heads and was of a fairly uniform dark greenish colour. The results in different trays were still erratic and investigation showed that aeration had not been uniform. The quality of the crude citromycetin was very much superior to that obtained in Tank 2. |
| 4 | Glucose | 8.5.26 | 3 trays on 18.5.26 4 trays on 19.5.26 5 trays on 20.5.26 | 10 11 12 | Ditto | Ditto | 0.11–0.35 | — | 131.3 gm. | The yield of citromycetin in the different trays still varied very considerably although in this tank an attempt was made to secure uniform and ample aeration. The quality of crude citromycetin was quite good. |
| 5 | Glucose | 25.5.26 | 6 trays on 1.6.26 6 trays on 2.6.26 | 10 11 | Ditto | Ditto | 0.15–0.4 | 223.3 | 142.2 gm. | In this tank each tray was sown with the spores from two Roux bottles instead of the customary one. Even as early as the fourth day the mycelium of all trays visible was this time seen to be green. The trays were more uniform than previously. It was definitely shown by analysis that very little citromycetin is left in the acidified and evaporated solution after standing for some time. |
| 6 | Glucose | 24.12.26 | 2 trays on 6.1.27 3 trays on 8.1.27 1 tray on 10.1.27 3 trays on 12.1.27 3 trays on 14.1.27 | 13 15 17 19 21 | Ditto | Ditto | nil–0.180 (average 0.095) | 107–236 (average 164.3) | 84.6 gm. | Sowing consisted of one Roux bottle per tray. In this tank for the first and only time aluminium grids were placed in each of the trays to support the mycelial felt. The poor results and small yields of citromycetin appear to be due in part at any rate to the presence of aluminium grids, since considerable quantities of potash alum crystals separated from the concentrated mother liquors on standing indicating that considerable erosion of the aluminium grids must have taken place. |
| 7 | Glucose | 12.2.27 | 6 trays on 22.2.27 5 trays on 23.2.27 1 tray on 24.2.27 | 10 11 12 | Ditto | Ditto | 0.11 (average of 12 trays) | 247.1 (average of 12 trays) | 125.7 gm. | The different trays gave more uniform results than previously with the exception of one tray in which growth was not quite so good as in the remainder. The quality of citromycetin obtained was quite good. |
| 8 | Glucose | 19.3.27 | 1 tray on 31.3.27 11 trays on 2.4.27 | 12 14 | Ditto | Ditto | 0.17 (average sample of all 12 trays) | 120.0 (average of 12 trays) | 70 gm. | This tank gave entirely unsatisfactory results for which no explanation has been forthcoming, but the unsatisfactory nature of it is evidenced not only by the low yield of crude citromycetin isolated but also by the estimated figure with alkaline iodine. |
| 9 | Glucose | 2.4.27 | 12 trays on 18.4.27 | 16 | Ditto | Ditto | See remarks | See remarks | 25.5 gm. See remarks. | In experiments 9–12 the new aluminium tank was used whereas in experiments 1–8 the old iron tank constructed in the factory had been used throughout. On opening the tank on 18.4.27 it was seen that only one tray had given a really typical growth with a characteristic colour reaction. Three others were fairly satisfactory but by no means good. These four trays were worked up in the usual way and gave a yield of 25.5 gm. of crude citromycetin. All the other trays gave little or no ferric chloride reaction and microscopic examination revealed the fact that they were all infected by extraneous organisms. These eight trays were therefore rejected and were not worked up. The cause of infection seems to have been splashing of the wash water from the air wash bottle into the trays. |
| 10 | Glucose | 29.4.27 | 12 trays on 10.5.27 | 11 | Ditto (average temperatures 24°–26° C.) | Ditto | 0.018 (average sample) | 289.8 (average sample) | 187 gm. | This tank was uniformly good throughout and gave the best yields and best quality of material so far obtained. The method of aeration adopted was to leave the tank for three days after inoculation with cotton wool plugs in the sampling holes. Aeration was carried out simultaneously through the main perforated pipe and through the twelve side tubes. At no time were any of the trays aerated separately and aeration of all trays was continuous from 2.5.27 to 10.5.27. The rate of aeration was about 50 cubic feet per hour. The sowing consisted of a suspension of spores from six Roux bottle cultures on beer wort agar. These were half of twelve sown on 25.3.27, the other six having been used for Tank 9. The six cultures used for Tank 10 had been stored from 2.4.27 till the date of using in an unused incubator at room temperature with the necks of the Roux bottles plugged but uncovered. |
| 11 | Glycerol (Price's pure) | 21.11.27 | 6 trays on 15.12.27 6 trays on 17.12.27 | 24 26 | New Biochemical Lab. (Average temperature about 18° C.) Ditto | 6 silica trays. 6 enamelled iron trays | — — | 400.2 186.9 | 117.0 gm. 58 gm. | In this tank six fused quartz trays referred to on p. 213 were used for the first time and in the other half of the tank six of the old enamelled iron trays were used. The results obtained with the silica trays were very much better than those obtained with the enamelled iron trays. The yield of citromycetin was almost twice as large, amounting for a full tank to the equivalent of 234 gm. The quality of the crude citromycetin was very much superior and the colour of the metabolism solution in the silica trays was a dark orange compared with a brownish black in the iron trays. This indicates that the mould is attacking and dissolving the enamel and the metal of this type of tray with deleterious results to the yield and quality of citromycetin. The appearance of the mycelium at the end of the experiment is quite different from that usually obtained on a glucose medium. On glucose this mould spores freely giving a dark green felt, whereas in a glycerol medium the mycelium is practically white at the end of the experiment. |
| 12 | Glycerol (Price's pure) | 22.12.27 | 6 trays on 17.1.28 6 trays on 18.1.28 | 26 27 | Ditto Ditto | 6 silica trays 6 enamelled iron trays | — — | 326.3 251.2 | 89.4 gm. 70.9 gm. | The results obtained with Tank 12 were very similar to those from Tank 11 except that the difference in yield between the silica and enamelled iron trays was not quite so marked. |



Various methods were tried to remove this tarry product and the two most suitable are described in the following methods of purification.

(a) 97 gm. of crude material from Tank 5, Fraction 5*b*, were dissolved in 1 litre of boiling 66 O.P. rectified spirit. The solution was cooled and while still slightly warm 3 litres of ether were added. This gave rise to a copious amorphous precipitate, consisting of the tarry material, which was filtered off after a few minutes. Weight of precipitate, 3.20 gm. The clear ether-alcohol solution was evaporated to about 500 c.c. when an equal volume of boiling water was added to the boiling solution and the mixture allowed to stand for two days. About 70 gm. of material separated in the form of dark yellow rhomboidal crystals. These were fractionally crystallised from 50 per cent. aqueous alcohol after treatment with a small quantity of charcoal. Crystallisation of the citromycetin was now very rapid, as it separated immediately on cooling in fine yellow needles. No other crystalline product was isolated, and the material was separated into four fractions of slightly decreasing purity:—

| | | | | | | | Gm. |
|------------|----|----|----|----|----|----|------|
| Fraction 1 | .. | .. | .. | .. | .. | .. | 38.8 |
| Fraction 2 | .. | .. | .. | .. | .. | .. | 7.2 |
| Fraction 3 | .. | .. | .. | .. | .. | .. | 14.6 |
| Fraction 4 | .. | .. | .. | .. | .. | .. | 3.3 |

The final mother-liquors on evaporation gave an alcohol-soluble tar weighing 24.8 gm. from which further quantities of citromycetin could be extracted by dissolving it in alcohol and precipitating with a large volume of ether.

(b) The tarry matter may also be separated somewhat less thoroughly by taking advantage of the fact that it is more soluble in 50 per cent. aqueous alcohol than is citromycetin. This method can only be applied efficiently, however, if the proportion of tar in the crude citromycetin is not high. With very impure specimens of crude citromycetin, the whole product is soluble in small amounts of aqueous alcohol and, for purification, is better treated by method (a). Method (b) is carried out as follows:—

A quantity of crude citromycetin is ground in a mortar with sufficient hot 50 per cent. aqueous alcohol to convert it into a thin paste. This is then quickly filtered on a Buchner funnel and washed with small quantities of the same solvent. The filtrate is brownish black in colour while the colour of the insoluble material is changed from the original dark brown to a much lighter yellowish brown. If the colour of the product is not satisfactory it is again extracted by grinding with hot aqueous alcohol. Finally, it is recrystallised several times from 50 per cent. aqueous alcohol, from which it separates quickly, on cooling, in yellow needles.

It should be noted that the use of animal or blood charcoal for purification of citromycetin solutions should be avoided whenever possible since citromycetin is so easily adsorbed by all samples of charcoal that excessive loss is inevitable if this material is used.

General properties of citromycetin.

Citromycetin crystallises from 50 per cent. aqueous alcohol in lemon-yellow needles. It is fairly soluble in cold absolute alcohol, hot glacial acetic acid, is less soluble in acetone, very slightly soluble in boiling water, and almost insoluble in cold water. It does not contain nitrogen.

It does not give a very definite melting point. On heating it begins to darken in colour at 263° C., and it becomes progressively darker in colour until it is almost black and then melts and decomposes at about 283°–285° C.

It is readily soluble in aqueous solutions of sodium carbonate or bicarbonate, with evolution of CO₂ and the formation of a deep yellow solution. It is also soluble in aqueous solutions of potassium acetate. On acidifying an aqueous solution of citromycetin in carbonate or acetate a yellow gel is formed which, on standing, slowly crystallises in yellow needles. It is immediately soluble in sodium hydroxide, giving a yellow to brown coloured solution which darkens on exposure to air, obvious decomposition taking place.

Its aqueous or alcoholic solutions give a very intense olive green colour with ferric chloride, which changes on standing to a dark brown. It reduces ammoniacal silver nitrate in the cold, and BENEDICT'S copper solution on boiling. It yields a green fluorescent solution with concentrated sulphuric acid.

It is optically inactive. 0.6484 gm. of air-dried citromycetin (C₁₄H₁₀O₇·2H₂O) dissolved in the theoretical amount of N/10 sodium hydroxide to give a monosodium salt and made up to 100 c.c. gave no rotation in a 40 cm. tube.

It does not contain any methoxyl groups, as shown by a ZEISEL estimation.

Citromycetin does not appear to contain a normal-reacting ketonic group, as several attempts to prepare one or other of the usual ketonic derivatives failed. The only product which could be isolated from the action of phenylhydrazine on citromycetin was a salt of phenylhydrazine with citromycetin which immediately decomposed with excess of either alkali or acid. In order to avoid the acidity of citromycetin itself attempts were made to prepare derivatives of the methyl ester of *O*-dimethylcitromycetin (see p. 225) with (a) phenylhydrazine, (b) semicarbazide, (c) hydroxylamine, (d) hydrazine. Varied conditions and durations of boiling were tried, but in no case were any derivatives recovered except in one experiment where the ester was boiled with alcoholic hydrazine for 20 hours. In this case a small amount of reaction product was isolated but this was definitely proved to be neither a hydrazone of the methyl ester of *O*-dimethylcitromycetin nor of *O*-dimethylcitromycetin itself.

Citromycetin, when crystallised from 50 per cent. aqueous alcohol, contains two molecules of water of crystallisation. This water of crystallisation is not easily removed. Long continued drying *in vacuo* over concentrated sulphuric acid or phosphorus pentoxide only removes a portion of it, and in order to ensure complete removal it is necessary to dry the material for some hours at a temperature of 150° C. Figures obtained on drying are summarized along with the combustion results (*q.v.*).

Considerable difficulty was met with for some time in obtaining consistent combustion results. The reasons for this were :—

- (a) Difficulty of removing all the water of crystallisation from citromycetin.
- (b) Difficulty of freeing citromycetin from traces of the decarboxylated product.

Acid solutions of citromycetin on heating readily lose CO_2 (see pp. 227 and 230) and this applies even to solutions to which acid has not been added, since the acidity of citromycetin itself is sufficient to cause a considerable amount of auto-decomposition. In the following combustion results these two sources of error have been eliminated as far as possible.

Three different samples were analysed and were purified as follows :—

(a) *By treatment with absolute alcohol*.—A sample of citromycetin which had been recrystallised several times from 50 per cent. alcohol was boiled with insufficient absolute alcohol to dissolve all of it. It was then filtered, refluxed for a few moments with charcoal, an equal volume of boiling water added, refiltered and the solution cooled quickly. The crystals which separated were again treated in a similar manner and were finally air-dried for analysis.

(b) *Via barium salt*.—A sample of citromycetin which had been several times crystallised from 50 per cent. alcohol was suspended in cold water and N/4 barium hydroxide added, with constant shaking, until about two-thirds of the material had dissolved. It was then filtered quickly and the clear filtrate immediately acidified with N/1 hydrochloric acid. Citromycetin separated quickly, first as a partial gel, which then crystallised completely in fine yellow needles. These were well washed and air-dried for analysis.

(c) *Via potassium salt*.—A quantity of the potassium salt of citromycetin was prepared according to the method given on p. 222. It was thoroughly extracted with boiling absolute alcohol, and 2.38 gm. were dissolved in the cold in 50 c.c. of water. This solution was filtered and to it were added 50 c.c. of cold alcohol containing the equivalent of 5 c.c. of N/1 hydrochloric acid. Citromycetin quickly crystallised, and was well washed and air-dried for analysis.

Before combustion all samples were dried to constant weight in a stream of dry, oxygen-free nitrogen at 150°C . By this means there was very little darkening in colour, whereas if the substance were dried in air at this temperature considerable darkening took place.

The following results were obtained on combustion of these dried samples. They agree with an empirical formula for citromycetin of $\text{C}_{14}\text{H}_{10}\text{O}_7 \cdot 2\text{H}_2\text{O}$. (Table VI, p. 220.)

The following attempts were made to determine the molecular weight of citromycetin :—

(a) *By the elevation of the boiling point of alcohol*.—0.2205 gm. of air-dried citromycetin was dissolved in 14.98 c.c. of absolute alcohol. Observed rise in boiling point of alcohol

TABLE VI.—Results of combustion of citromycetin.

| Sample. | Weight of Air Dried Material taken. | Loss in Weight at 150° C. | Percent-age of H ₂ O of cryst. | Weight of Sample (dried at 150° C.) analysed. | Weight, CO ₂ . | Weight, H ₂ O. | Percent-age, C. | Percent-age, H. |
|--|-------------------------------------|---------------------------|---|---|---------------------------|---------------------------|-----------------|-----------------|
| | gm. | gm. | | gm. | gm. | gm. | | |
| Purified according to method (a) ... | 0.1637 | 0.0178 | 10.87 | 0.1459 | 0.3091 | 0.0468 | 57.78 | 3.59 |
| Purified according to method (b) ... | 0.1494 | 0.0174 | 11.64 | 0.1320 | 0.2810 | 0.0420 | 58.02 | 3.56 |
| Purified according to method (c) ... | 0.1503 | 0.0161 | 10.71 | 0.1342 | 0.2846 | 0.0420 | 57.83 | 3.59 |
| Theoretical for C ₁₄ H ₁₀ O ₇ · 2H ₂ O | — | — | 11.04 | — | — | — | — | — |
| C ₁₄ H ₁₀ O ₇ ... | — | — | — | — | — | — | 57.92 | 3.47 |

= 0.107° C., corresponding to a molecular weight of 199.5 (theoretical for C₁₄H₁₀O₇ · 2H₂O = 326).

(b) *By Barger's capillary tube method*, BARGER (1904).—A solution of citromycetin in alcohol was compared by the above method against (1) a solution of vanillin in alcohol, and (2) a solution of dichloroaniline in alcohol. In each case a molecular weight of about 240 was observed.

The low figure for the molecular weight of citromycetin as obtained by methods (a) and (b) is probably caused by the ionisation of citromycetin in alcohol, since it is a strong acid.

(c) *By titration against sodium hydroxide*.—0.4728 gm. of air-dried citromycetin was suspended in a little water and titrated with N/10 sodium hydroxide to phenolphthalein. The sodium hydroxide was run in, with constant shaking, and the citromycetin slowly dissolved giving a bright yellow solution. The first colour change from yellow to orange was noticed after 28.33 c.c. of N/10 sodium hydroxide solution had been added, and a definite pink colour, confirmed by external indicator, after the addition of 28.78 c.c. These figures correspond to an equivalent for citromycetin of 166.8 and 164.3 respectively, and if one assumes that citromycetin is dibasic they give a molecular weight of 333.6 and 328.6 respectively. (Theoretical for C₁₄H₁₀O₇ · 2H₂O = 326.)

It is interesting to note that on the addition of N/10 hydrochloric acid to the titrated solution nothing visible occurred until after the addition of more than 14.4 c.c. of N/10 acid, indicating that the monosodium salt of citromycetin is readily soluble in water. On the addition of further N/10 acid citromycetin began to be precipitated and finally separated as a yellow gel which on standing shrank to a mass of yellow needles.

In a similar experiment with 0.4259 gm. of citromycetin dried to constant weight in

nitrogen at 150°C, part of the fraction purified via the barium salt (see p. 219) gave titration figures of 29·93 c.c. of N/10 sodium hydroxide to the first change and 30·01 c.c. to a permanent pink, corresponding to molecular weights of 284·6 and 283·9. (Theoretical for $C_{14}H_{10}O_7 = 290$.)

Derivatives of citromycetin.

(1) *Diacetyl compound* $C_{14}H_8O_5 (O.CO.CH_3)_2$.—The diacetyl derivative has been prepared by two different methods.

(a) By treatment of the potassium salt of citromycetin with acetic anhydride.

10·01 gm. of the dried potassium salt prepared from 10 gm. of citromycetin by the method described on p. 222 were mixed with 50 c.c. of acetic anhydride and allowed to stand overnight at room temperature. In the morning the potassium salt had completely dissolved. (On long standing in a sealed tube typical crystals of the acetyl derivative are deposited.) The solution was evaporated to complete dryness in a vacuum desiccator. The dried residue dissolved completely in 50 c.c. of water. Addition of dilute hydrochloric acid to this produced a heavy precipitate almost white in colour, which was filtered off, washed and dried. Weight = 10·18 gm. It was recrystallised from absolute alcohol, from which it separates in white prismatic crystals.

(b) By the usual method with acetic anhydride and sodium acetate.

5 gm. of citromycetin were heated with 5 gm. of anhydrous sodium acetate and 15 c.c. of acetic anhydride for one hour. The cooled residue was completely soluble in cold water, and this solution, on acidification with hydrochloric acid, gave a heavy white precipitate. This was dried and recrystallised from absolute alcohol. Yield of recrystallised material = 3·3 gm. of an acetyl compound identical with that prepared from the potassium salt. This substance had a melting point of 223°–224° C. (with decomposition). Although insoluble in water it is a strong acid which is readily soluble in aqueous solutions of sodium acetate or of potassium bicarbonate with evolution of carbon dioxide. Its alcoholic solution gives a pale yellow colour with ferric chloride.

The diacetyl compound shows a curious and so far unexplained behaviour on recrystallisation. It crystallises from absolute alcohol in typical “coffin lid” shaped crystals having a melting point of 223°–224° C. (with decomposition). On the other hand it separates from its solution in aqueous potassium acetate on acidification with dilute hydrochloric acid in needles having a melting point of 235°–236° C. (with decomposition). Neither of these forms contains any water of crystallisation and they are readily interconvertible, so that needle-shaped crystals (melting point 236° C.) on crystallising from absolute alcohol separate as “coffin lid” crystals with a melting point of 223° C., and these can be reconverted into the needle-shaped crystals by dissolving in aqueous potassium acetate and precipitating with dilute hydrochloric acid.

Combustions agree with the formula $C_{14}H_8O_5 (O.CO.CH_3)_2$, i.e., $C_{18}H_{14}O_9$.

This material was dried to constant weight at 100° C. in air before combustion.

Results of combustion of the diacetyl derivative of citromyccetin.

| Weight of Sample. | Weight of CO ₂ . | Weight of Water. | Percentage Carbon. | Percentage Hydrogen. |
|--|-----------------------------|------------------|--------------------|----------------------|
| 0.1678 | 0.3556 | 0.0593 | 57.78 | 3.96 |
| 0.1555 | 0.3292 | 0.0548 | 57.74 | 3.94 |
| Theoretical for C ₁₈ H ₁₄ O ₉ | — | — | 57.75 | 3.77 |

The compound was proved to be a diacetyl compound by determining the amount of acetic acid produced on hydrolysis. 0.4081 gm. was boiled for three hours under a reflux condenser with 30 c.c. of N/10 sulphuric acid and then heated for a further six hours on a steam bath. At the end of hydrolysis the acetic acid was distilled off by evaporation *in vacuo* until completely removed. The acetic acid produced was equivalent to 21.86 c.c. of N/10 sodium hydroxide. (Theoretical for C₁₄H₈O₅ (O.CO.CH₃)₂ is 21.83 c.c.)

During hydrolysis the colour of the liquid rapidly became golden yellow, and a quantity of yellow needle crystals separated. These were composed of decarboxylated citromyccetin, *i.e.*, of "citromycin" (see p. 227). At the same time carbon dioxide was evolved and was measured in one experiment as follows:—0.5040 gm. of diacetyl citromyccetin was hydrolysed with 50 c.c. of 2N sulphuric acid on a boiling water bath for 17 hours. A constant stream of nitrogen was passed through the hydrolysis flask and reflux condenser and was bubbled through N/4 barium hydroxide, the excess of which was titrated at the end of the experiment with N/2 hydrochloric acid. Carbon dioxide equivalent to 5.21 c.c. of N/2 hydrochloric acid was formed. (Theoretical for 0.5040 gm. of diacetylcitromyccetin — CO₂ ≡ 5.39 c.c. N/2 HCl). The products of hydrolysis of diacetylcitromyccetin are thus two molecules of acetic acid, one molecule of carbon dioxide and one molecule of citromycin (see p. 227).

(2) *Potassium salt* (C₁₄H₈O₇K.H₂O).—The potassium salt is readily prepared by adding an alcoholic solution of citromyccetin to an alcoholic solution of potassium acetate. 10 gm. of pure citromyccetin were dissolved in 400 c.c. of boiling absolute alcohol, filtered, and to the boiling solution were added 100 c.c. of a filtered solution in absolute alcohol of 5 gm. of anhydrous potassium acetate. Crystallisation of the potassium salt commenced almost immediately. The potassium salt was filtered when cold, well washed with absolute alcohol and air-dried. Yield of potassium salt = 10.01 gm. The potassium salt crystallises in canary yellow needles, sometimes slightly curved, and showing a characteristic sprouting effect at the ends of the crystals.

0.6662 gm. of the air-dried potassium salt, dried to constant weight at 150° C. in nitrogen, lost 0.0356 gm. of water corresponding to 5.34 per cent. Calculated for C₁₄H₈O₇K.H₂O = 5.20 per cent. A sample of the potassium salt dried to constant weight in nitrogen at 150° C. gave the following combustion results.

| Weight of Sample Analysed. | Total weight of CO ₂ | Weight of Water. | Weight of Ash (K ₂ CO ₃). | Percentage Carbon. | Percentage Hydrogen. | Percentage Potassium. |
|---|---------------------------------|------------------|--|--------------------|----------------------|-----------------------|
| 0·1683 gm. | 0·3178 | 0·0453 | 0·0351 | 51·50 | 3·01 | 11·79 |
| 0·1632 gm. | 0·3087 | 0·0428 | 0·0344 | 51·60 | 2·93 | 11·93 |
| Calculated for C ₁₄ H ₉ O ₇ K. ... | — | — | — | 51·20 | 2·76 | 11·91 |

0·6298 gm. dried to constant weight at 150° C. and ignited with concentrated H₂SO₄ gave 0·1661 gm. K₂SO₄ corresponding to 11·82 per cent. K.

(3) *Barium salt* (C₁₄H₉O₇)₂Ba · 3H₂O.—A weighed quantity (about 1 gm.) of citromycetin was suspended in 40 c.c. of cold water, and to this was added, with constant shaking, the calculated volume of N/4 barium hydroxide necessary to neutralise the weighed amount of citromycetin, on the assumption that this is a monobasic acid. Most of the citromycetin dissolved, and the solution was filtered as quickly as possible from the small residue of citromycin. On standing the barium salt crystallised from the clear filtrate in orange plates. These were filtered off, washed, dried and analysed.

0·1432 gm. of barium salt dried to constant weight in nitrogen at 150° C. lost 0·0102 gm. water, equivalent to 7·11 per cent. (Theoretical for (C₁₄H₉O₇)₂Ba · 3H₂O = 7·02 per cent.)

Samples of the barium salt dried to constant weight in nitrogen at 150° C. gave the following combustion results:—

| Weight of Sample. | Total weight of CO ₂ | Weight of Water. | Weight of Ash (BaCO ₃). | Percentage Carbon. | Percentage Hydrogen. | Percentage Barium. |
|---|---------------------------------|------------------|-------------------------------------|--------------------|----------------------|--------------------|
| 0·1326 gm. | 0·2300 | 0·0340 | 0·0366 | 47·30 | 2·87 | 19·20 |
| 0·1473 gm. | 0·2539 | 0·0379 | 0·0419 | 47·00 | 2·88 | 19·77 |
| Calculated for (C ₁₄ H ₉ O ₇) ₂ Ba ... | — | — | — | 46·96 | 2·53 | 19·20 |

(4) *Hydrobromide* C₁₄H₁₀O₇ · HBr.—1 gm. of citromycetin was dissolved in 40 to 50 c.c. of hot glacial acetic acid, the solution filtered, boiled, and to this was added 1–2 c.c. of fuming hydrobromic acid. The colour of the solution changed immediately from bright yellow to a dark orange and on standing the hydrobromide crystallised in dark orange prisms. These were washed with glacial acetic acid and dried *in vacuo* over sulphuric acid and potassium hydroxide. Weight = 1·01 gm. The hydrobromide is immediately decomposed by water giving citromycetin and hydrobromic acid.

0·5131 gm. of the hydrobromide was suspended in a little water and titrated with N/10 sodium hydroxide solution, using phenolphthalein as indicator. The titration was

carried out in the same manner as that described for the titration of citromycetin on p. 220. 41.82 c.c. of N/10 sodium hydroxide were required for neutralisation. (Theory for $C_{14}H_{10}O_7 \cdot HBr$, assuming this titrates as a tribasic acid, is 41.49 c.c.)

The percentage of hydrobromic acid in the hydrobromide was estimated by suspending a weighed amount in water, filtering from the precipitated citromycetin and estimating the HBr in the filtrate and washings by precipitation as AgBr after acidification with nitric acid.

| Weight of Hydrobromide. | Weight of AgBr. | Percentage of HBr. |
|---|-----------------|--------------------|
| gm. | gm. | |
| 0.4877 | 0.2482 | 21.93 |
| 0.5131 | 0.2604 | 21.86 |
| Theoretical for $C_{14}H_{10}O_7 \cdot HBr$ | — | 21.81 |

When citromycetin is dissolved in hot fuming *aqueous* hydrochloric, hydrobromic or hydriodic acid, and the solutions allowed to cool, citromycetin compounds separate in each case. A particularly promising compound is obtained with hydriodic acid and a quantity of this was prepared.

1 gm. of citromycetin was boiled with 100 c.c. of constant boiling point hydriodic acid solution until dissolved and then left to cool slowly. The hydriodide separates in beautiful, very fine orange needles about $\frac{1}{4}$ in. long. These were filtered off on a Gooch crucible, washed with glacial acetic acid and then with dry ether. The yield of hydriodide was 1.15 gm. The crystals were dried *in vacuo* over phosphorus pentoxide and analysed in a similar manner to the hydrobromide. In duplicate experiments 0.2009 gm. and 0.2016 gm. gave 0.1224 gm. and 0.1231 gm. AgI respectively, corresponding to 33.20 per cent. and 33.26 per cent. HI. (Theoretical for $C_{14}H_{10}O_7 \cdot HI = 30.62$ per cent.) This compound is obviously not a simple hydriodide of citromycetin, although it may be the hydriodide of citromycin (p. 229) $C_{13}H_{10}O_5 \cdot HI$. (Theoretical percentage of HI = 34.21.) It separates in the same typical orange crystals from the boiling hydriodic acid used in all the ZEISEL estimations subsequently described, *e.g.*, from the methyl ester of *O*-dimethylcitromycetin, *O*-dimethylcitromycetin itself, *O*-dimethylcitromycin (see p. 230) and *O*-monomethylcitromycin. It is immediately decomposed by treatment with water and the material regenerated is quite different in appearance from citromycetin. The regenerated material gives an instantaneous brown colour with ferric chloride in alcoholic solution (*cf.* citromycin), and crystallises from 50 per cent. aqueous alcohol in bunches of pale yellow feathery needles.

0.1579 gm. was dried in nitrogen at 150° C. There was no loss in weight (*cf.* citromycetin). On combustion this weight of material gave 0.3719 gm. CO_2 and 0.0606 gm. water, corresponding to 64.23 per cent. carbon and 4.29 per cent. hydrogen.

(Theoretical for citromycin, $C_{13}H_{10}O_5$, carbon = 63·39 per cent., hydrogen = 4·09 per cent.)

(5) *The methyl ester of O-dimethylcitromycetin* $C_{13}H_7O_3 (OCH_3)_2 \cdot COOCH_3$.—20 gm. of citromycetin were suspended in 200 c.c. of water and treated with 100 c.c. of dimethyl sulphate. 10 per cent. sodium hydroxide was added with constant shaking, keeping the reaction mixture just alkaline. The temperature of the reacting mixture was regulated at about 40° C. The reaction was complete in about two hours. The methyl ester separated in a crystalline form towards the end of the methylation, and was separated and recrystallised several times from 50 per cent. alcohol, using charcoal to clear the solution. Yield of recrystallised material = 12 gm.

Large quantities of this compound were subsequently made for the investigation of the products of alkaline hydrolysis and were prepared by methylating the crude citromycetin as isolated from the metabolism solution. 64 gm. of crude citromycetin gave 31 gm. of pure methyl ester.

This compound (*O*-dimethylcitromycetin methyl ester) is also produced when citromycetin is methylated by treatment with diazomethane in ether solution.

The methyl ester crystallises in white silky needles having a melting point of 178° C. without decomposition. It is readily soluble in hot absolute or 50 per cent. alcohol but is almost insoluble in the cold. It is insoluble in aqueous solutions of potassium acetate, potassium bicarbonate or even of potassium hydroxide. Its alcoholic solution gives no colour with ferric chloride.

In view of the fact that this compound is very stable and can be readily purified by crystallisation, a sample was sent to SCHOELLER (Berlin) for analysis in order to obtain external confirmation of the empirical formula for citromycetin suggested by our own combustion results on this material.

The sample analysed was dried to constant weight over P_2O_5 *in vacuo* and gave the following results :—

| Weight of Substance. | Weight of CO_2 . | Weight of Water. | Percentage Carbon. | Percentage Hydrogen. |
|--|--------------------|------------------|--------------------|----------------------|
| | mg. | mg. | | |
| 4·770 mg. | 10·735 | 2·10 | 61·41 | 4·93 |
| 4·392 mg. | 9·885 | 1·97 | 61·40 | 5·02 |
| Theoretical for $C_{13}H_7O_3 \cdot (OCH_3)_2 \cdot COOCH_3$ | — | — | 61·43 | 4·86 |

ZEISEL estimations of the methoxyl content gave the following results :—0·3095 gm. of ester gave 0·6439 gm. AgI, corresponding to 27·47 per cent. OCH_3 in the ester. In a duplicate estimation 0·2034 gm. ester gave 0·4321 gm. AgI, corresponding to 28·03 per cent. OCH_3 . (Theoretical for $C_{13}H_7O_3 \cdot (OCH_3)_2 \cdot COOCH_3$ = 28·02 per cent.)

(6) *O-Dimethylcitromycetin* $C_{13}H_7O_3 \cdot (OCH_3)_2 \cdot COOH \cdot H_2O$.—*O*-Dimethylcitromycetin

was prepared from the alkaline filtrate obtained when the crude methyl ester of *O*-dimethylcitromyccetin was filtered off from the methylation mixture. The details are as follows:—4 gm. of citromyccetin were shaken with 40 c.c. of water and dissolved by adding 24.5 c.c. of N/1 sodium hydroxide. 20 c.c. of methyl sulphate were then added and the methylation carried out as described above. About 160 c.c. of N/1 sodium hydroxide were added during the methylation. It was noticed that the crystalline precipitate of the methyl ester which appears towards the end of the methylation seems to decrease in amount as more sodium hydroxide is added, and if it is desired to prepare *O*-dimethylcitromyccetin the best yields of this material are obtained by keeping the reaction mixture alkaline for some considerable time. If, on the other hand, it is desired to prepare the methyl ester it is advisable to leave this in contact with excess of sodium hydroxide for as short a time as possible. In the present experiment the exposure to alkali was somewhat prolonged, 1.59 gm. of crude methyl ester were filtered off and the alkaline filtrate, on acidification, gave 2.46 gm. of crude *O*-dimethylcitromyccetin. This was repeatedly crystallised from 50 per cent. aqueous alcohol, from which *O*-dimethylcitromyccetin separates in white prisms which melt with decomposition at 217°–218° C. The substance is soluble in aqueous solutions of potassium acetate and potassium bicarbonate, and its alcoholic solution gives only a yellow colour with ferric chloride.

0.1497 gm. of the air-dried material, dried to constant weight in nitrogen at 140° to 150° C. loses 0.0079 gm. = 5.28 per cent. (Theoretical percentage of H_2O in $\text{C}_{13}\text{H}_7\text{O}_3 \cdot (\text{OCH}_3)_2 \cdot \text{COOH} \cdot \text{H}_2\text{O} = 5.36$.)

0.1418 gm. dried to constant weight at 150° C. in nitrogen gave in a ZEISEL estimation 0.2089 gm. $\text{AgI} = 19.46$ per cent. OCH_3 . (Theoretical percentage of OCH_3 in $\text{C}_{13}\text{H}_7\text{O}_3 \cdot (\text{OCH}_3)_2 \cdot \text{COOH} = 19.50$.)

(7) *Action of bromine on citromyccetin*.—1 gm. of citromyccetin was dissolved in 5 c.c. of glacial acetic acid, cooled in ice, and 0.8 c.c. of bromine added. Orange red crystals very soon separated and there was considerable evolution of hydrobromic acid. The crystals were filtered off after standing overnight, washed with glacial acetic acid and dried *in vacuo* over potassium hydroxide. Weight of crystals = 1.60 gm. The bromine compound consists of orange red prisms which it was not found possible to recrystallise because of decomposition in all solvents tried. The substance was immediately decomposed by water, giving rise to a very dark brownish black powder. 0.2885 gm. of the bromine compound was treated with water and, after standing for half an hour, the insoluble material was filtered off and the amount of hydrobromic acid in combined filtrate and washings estimated as silver bromide. The weight of silver bromide was 0.2621 gm., which corresponds to 38.7 per cent. of bromine split off as HBr from the bromine compound. This figure was confirmed in a separate experiment by titration of the hydrobromic acid with sodium hydroxide, giving a figure of 39.1 per cent. On account of the unpromising nature of the compound it was not further investigated, but it appears that the action of bromine on citromyccetin first produces substitution and that the hydrobromic acid thus liberated then combines with the

brominated compound to give a hydrobromide. Treatment of this compound with water not only splits off the hydrobromic acid from the hydrobromide linking but must also produce hydrobromic acid from at least one of the substituted bromine atoms. This would explain the high percentage of hydrobromic acid split off on treatment with water.

(8) *Citromycin*. $C_{13}H_{10}O_5$.—On boiling citromycetin with dilute acid, carbon dioxide is slowly given off and a new compound, citromycin, is produced. The course of this reaction was investigated quantitatively as follows:—0.3115 gm. of citromycetin, dried in nitrogen at $150^\circ C$., was boiled in an atmosphere of nitrogen with 50 c.c. of 2N sulphuric acid. A slow stream of purified nitrogen was bubbled through the reaction mixture and up through an upright condenser. The gaseous products were then bubbled through standard barium hydroxide. Hydrolysis was almost complete in 12 hours, but was continued for a total of 18 hours in all. The barium hydroxide was then titrated against N/2 hydrochloric acid to phenolphthalein. Carbon dioxide equivalent to 4.71 c.c. of N/2 hydrochloric acid had been produced. (Theoretical for $C_{14}H_{10}O_7 \rightarrow C_{13}H_{10}O_5 + CO_2 \equiv 4.30$ c.c. N/2 HCl.) 0.27 gm. of citromycin was recovered from the hydrolysis mixture. (Theoretical yield = 0.26 gm.) From this it appears that on acid hydrolysis one molecule of citromycetin loses one molecule of carbon dioxide to form one molecule of citromycin.

For the preparation of citromycin in quantity 20 gm. of citromycetin were boiled with 1,500 c.c. of 2N sulphuric acid for two days. The yellow crystalline product, weighing 14.9 gm., was recrystallised from 50 per cent. alcohol, from which it separates in yellow needles containing no water of crystallisation. Citromycin is somewhat paler in colour than citromycetin and is also less soluble in all solvents. On heating it begins to darken at 255° to $260^\circ C$., melting very indefinitely at 285° to $290^\circ C$. It is insoluble in aqueous solutions of sodium acetate or bicarbonate but is readily soluble in sodium hydroxide, giving an orange brown solution. Its solution in alcohol gives an intense brown colour with ferric chloride. It contains no methoxyl groups as was shown by a ZEISEL estimation.

It titrates as a monobasic acid to phenolphthalein. 0.2244 gm. was titrated with N/10 sodium hydroxide with phenolphthalein as indicator, and 9.17 c.c. of N/10 sodium hydroxide were required for neutralisation. (Theoretical for $C_{13}H_{10}O_5$ titrated as a monobasic acid = 9.12 c.c. N/10 NaOH.)

Analyses of citromycin, dried to constant weight at $150^\circ C$. in nitrogen, agree with the formula $C_{13}H_{10}O_5$.

| Weight of Sample. | Weight of CO_2 . | Weight of Water. | Percentage Carbon. | Percentage Hydrogen. |
|-----------------------------------|--------------------|------------------|--------------------|----------------------|
| 0.1685 gm. | 0.3905 | 0.0621 | 63.20 | 4.12 |
| 0.1186 gm. | 0.2761 | 0.0428 | 63.47 | 4.04 |
| 0.1039 gm. | 0.2411 | 0.0392 | 63.30 | 4.22 |
| 0.1010 gm. | 0.2345 | 0.0390 | 63.32 | 4.32 |
| Theoretical for $C_{13}H_{10}O_5$ | — | — | 63.39 | 4.09 |

(9) *Diacetylcitromycin*. $C_{13}H_8O_3(O.CO.CH_3)_2$.—2 gm. of citromycin were heated to boiling with 2 gm. of anhydrous sodium acetate and 6 c.c. of acetic anhydride in an oil bath for 30 minutes. The mixture was cooled and diluted with water. A quantity of crude acetyl compound separated and was filtered off and washed. Yield of crude material = 2.56 gm. (Note the fundamental difference between the behaviour of citromycetin and citromycin on acetylation. Diacetylcitromycetin remains in solution in the acetylation mixture, presumably because of the presence of a COOH group in citromycetin which is absent in citromycin.)

The crude acetyl compound was recrystallised from 50 per cent. aqueous alcohol from which it separated in fine white needles having a melting point of 221° – 222° C. There was slight darkening at the melting point but no other obvious decomposition. It is insoluble in aqueous solutions of potassium acetate or potassium bicarbonate and gives no colour reaction in alcoholic solution with ferric chloride.

It was shown that the substance was a diacetyl compound by the following means: 0.4037 gm. was hydrolysed by boiling with 25 c.c. of N/1 sulphuric acid for 12 hours. The hydrolysed mixture was left at room temperature for a week and the regenerated citromycin was then filtered off on a sintered glass funnel, washed and dried at 100° C. Weight of regenerated citromycin = 0.2980 gm. \equiv 73.8 per cent. (Theoretical for $C_{13}H_8O_3(O.CO.CH_3)_2 = 74.55$ per cent. and for $C_{13}H_7O_2(O.CO.CH_3)_3 = 66.10$ per cent.) The amount of acetic acid produced on hydrolysis was also estimated by the method given on p. 222. The acetic acid produced is equivalent to 23.59 c.c. of N/10 sodium hydroxide. (Theoretical for $C_{13}H_8O_3(O.CO.CH_3)_2 = 24.47$ c.c.)

Combustion results agree with the formula $C_{13}H_8O_3(O.CO.CH_3)_2$.

| Weight of Sample. | Weight of CO_2 . | Weight of Water. | Percentage Carbon. | Percentage Hydrogen. |
|---|--------------------|------------------|--------------------|----------------------|
| 0.1380 gm. | 0.3133 | 0.0546 | 61.90 | 4.43 |
| 0.1305 gm. | 0.2964 | 0.0552 | 61.95 | 4.73 |
| Theoretical for $C_{13}H_8O_3(O.CO.CH_3)_2$ | — | — | 61.80 | 4.27 |

(10) *Potassium salt of citromycin*. $C_{13}H_{10}O_5 \cdot C_{13}H_9O_5K$.—1 gm. of citromycin was dissolved in 100 to 120 c.c. of boiling absolute alcohol and to this were added 10 c.c. of 5 per cent. alcoholic potassium acetate solution. The colour of the solution immediately darkened from pale yellow to orange. After boiling for a few minutes the solution was filtered and left to crystallise. The potassium salt crystallised out slowly in the cold in dense clusters of orange needles. Yield = 0.54 gm. It is much more soluble in alcohol than the potassium salt of citromycetin and, unlike this, does not lose weight on drying at 150° C. in nitrogen.

For analysis 0.1955 gm. of the salt was acidified with 10 c.c. of N/1 sulphuric acid,

left overnight in the cold and the precipitated citromycin weighed on a sintered glass funnel. Weight of citromycin regenerated = 0.1806 gm. \equiv 92.39 per cent. The filtrate was evaporated to dryness and ashed in a platinum basin. Weight of K_2SO_4 = 0.0348 gm. \equiv 7.99 per cent. potassium. Since the theoretical values for citromycin and potassium in $C_{13}H_9O_5K$ are 86.27 per cent. and 13.76 per cent. respectively it seemed that this estimation might be incorrect. It was, however, confirmed by ashing 0.3418 gm. of the potassium salt directly with sulphuric acid. Weight of ash = 0.0555 gm. \equiv 7.29 per cent. K in potassium salt. The potassium salt of citromycin appears to be a compound containing two molecules of citromycin with only one hydrogen atom replaced by potassium. Such a compound $C_{26}H_{19}O_{10}K$ gives 92.84 per cent. citromycin and 7.36 per cent. potassium.

Similar compounds have already been described by PERKIN (1899). In this paper PERKIN describes the preparation of the potassium salts of a number of phenolic colouring matters by the interaction of an alcoholic solution of the colouring matter with an alcoholic solution of potassium acetate. The majority of the colouring matters tested by PERKIN give normal potassium salts, but rhamnetin and rhamnazin, the methyl ethers of quercetin, form potassium salts analogous to those of citromycin. Since each consists of a monopotassium derivative of a double molecule of the colouring matter, this property goes hand in hand with the relatively feeble acid properties of citromycin.

(11) *Hydrobromide of citromycin*. $C_{13}H_{10}O_5 \cdot HBr$.—1 gm. of citromycin was dissolved in 75 c.c. of boiling glacial acetic acid and to this were added, while boiling, 2 c.c. of fuming hydrobromic acid. The original pale yellow colour changed to orange. The hydrobromide separated in orange yellow needles with irregular splintered ends. After washing with glacial acetic acid it was dried over sulphuric acid and potassium hydroxide for analysis. Weight of hydrobromide = 1.27 gm.

The hydrobromide was analysed by treating a weighed amount with water, which decomposed it immediately into citromycin and hydrobromic acid. The citromycin was filtered on a sintered glass funnel, dried and weighed, and the hydrobromic acid in the filtrate estimated as silver bromide.

| Weight of Hydrobromide. | Weight of AgBr. | Weight of Regenerated Citromycin. | Percentage HBr. | Percentage Regenerated Citromycin. |
|---|-----------------|-----------------------------------|-----------------|------------------------------------|
| 0.2875 gm. | 0.1591 | 0.2128 | 23.83 | 74.00 |
| 0.2901 gm. | 0.1623 | 0.2151 | 24.10 | 74.15 |
| Theoretical for $C_{13}H_{10}O_5 \cdot HBr$. | — | — | 24.75 | 75.25 |

A hydriodide was prepared by dissolving citromycin in boiling concentrated hydriodic acid. On cooling, glistening orange needles separated which were identical with those prepared in a similar manner from citromycetin and hydriodic acid (see p. 224).

(12) *O-Dimethylcitromycin*. $C_{13}H_8O_3 \cdot (OCH_3)_2$.—*O*-Dimethylcitromycin was prepared by methylating citromycin with dimethyl sulphate in presence of sodium hydroxide. 2 gm. of citromycin were dissolved in 8 to 10 c.c. of N/1 sodium hydroxide and 10 c.c. of dimethyl sulphate added. The whole was shaken vigorously and kept just alkaline by additions of N/1 sodium hydroxide. The *O*-dimethyl derivative separated during the course of methylation and was filtered off, washed and dried. Weight of crude product = 1.58 gm. It was recrystallised several times from 50 per cent. aqueous alcohol and when pure separated in pale yellow polyhedra, having a melting point of 225°–227° C. It was insoluble in carbonates or sodium hydroxide solution and its alcoholic solution gave a negative test with ferric chloride. It did not lose weight on drying in nitrogen at 150° C. and a sample so dried gave the following result in a ZEISEL estimation: 0.1933 gm. gave 0.3267 gm. of AgI, corresponding to 22.32 per cent. OCH_3 . (Theoretical percentage OCH_3 in $C_{13}H_8O_3 \cdot (OCH_3)_2$ = 22.62 per cent.)

(13) *O-Monomethylcitromycin*. $C_{13}H_8O_3 \cdot (OH) \cdot (OCH_3)$.—The monomethyl compound was prepared by methylating citromycin with methyl iodide and alcoholic potassium hydroxide. 1 gm. of citromycin was heated with 2 gm. of methyl iodide (which is rather more than 3 molecules of CH_3I to 1 molecule of citromycin) and a solution of 0.79 gm. of potassium hydroxide in 6.6 c.c. of methyl alcohol, this being the amount of KOH necessary to decompose 2 gm. of CH_3I . The mixture was placed in a small Carius tube previously filled with nitrogen, and then sealed and heated in a boiling water bath for one hour. The mixture was diluted with water and the precipitated methyl derivative recrystallised from 50 per cent. aqueous alcohol. The *O*-monomethylcitromycin so prepared crystallises in small yellow needles having a melting point of 183–185° C. It is readily soluble in sodium hydroxide solution but insoluble in carbonates, and its alcoholic solution gives a yellow colour with ferric chloride, *i.e.*, a practically negative reaction.

When recrystallised from aqueous alcohol it appears to contain one molecule of water of crystallisation. 0.2020 gm., which had been previously dried *in vacuo* over sulphuric acid, was dried to constant weight in nitrogen at 140° C. Loss in weight = 0.0103 gm. corresponding to 5.10 per cent. of water. (Theoretical for $C_{13}H_8O_3 \cdot (OH) \cdot (OCH_3) \cdot H_2O$ = 6.48 per cent.)

0.1917 gm. of this dried material gave in a ZEISEL estimation 0.1877 gm. of AgI = 12.93 per cent. OCH_3 . (Theoretical for $C_{13}H_8O_3 \cdot (OH) \cdot (OCH_3)$ = 11.93 per cent.)

Decomposition products of citromycetin.

Section 1.—By acid hydrolysis.

As previously stated (p. 227) citromycetin on boiling with dilute sulphuric acid gives rise to one molecule of carbon dioxide and one molecule of citromycin. No other hydrolytic products have been found. 1 gm. of citromycetin was boiled with 150 c.c. of 2N sulphuric acid in an atmosphere of nitrogen with a reflux condenser for nine hours

until no further carbon dioxide was evolved. At the end of this time the hydrolysis liquid was distilled and the distillate tested:—

(a) For acetone, with sodium nitroprusside and ammonia (ROTHERA'S test), and with 2:4-dinitrophenylhydrazine. Both gave negative results.

(b) For acetic acid. This also gave a negative result.

Section 2.—With alkaline iodine.

When citromycetin is treated with alkaline iodine obvious decomposition takes place and iodoform is formed. Since oxidation with alkaline iodine has been applied successfully to the estimation of kojic acid (see Part VIII), the method was tested for its possible application to the estimation of citromycetin.

0.2900 gm. (M/100) of citromycetin, dried in nitrogen at 150° C. to constant weight, was dissolved in the minimum amount of N/10 sodium hydroxide and made up to 100 c.c. Portions of this were then treated with a known volume of N/10 iodine solution, and a definite volume of N/10 sodium hydroxide was added, drop by drop, during the space of three minutes. The mixed solutions were allowed to stand for two hours, and were then acidified with 20 c.c. of N/1 sulphuric acid and the excess of iodine titrated with N/10 sodium thiosulphate solution. The results are summarized in Table VII.

TABLE VII.—Reaction of citromycetin with alkaline iodine.

| c.c. of Citromycetin Solution used. | c.c. of Water added. | c.c. N/10 Iodine added. | c.c. N/10 NaOH added. | c.c. N/10 Thio. used. | c.c. N/10 Iodine absorbed per 10 c.c. of Citromycetin Solution. |
|-------------------------------------|----------------------|-------------------------|-----------------------|-----------------------|---|
| 2 | 98 | 40 | 50 | 38.44 | 6.55 |
| 5 | 95 | 40 | 50 | 35.88 | 7.74 |
| 10 | 90 | 40 | 50 | 32.09 | 7.66 |
| 15 | 85 | 40 | 50 | 27.89 | 7.91 |
| 20 | 80 | 40 | 50 | 24.02 | 7.87 |
| 15 | 35 | 20 | 25 | 8.17 | 7.81 |
| 20 | 30 | 20 | 25 | 5.49 | 7.20 |
| Blank experiment | 100 | 40 | 50 | 39.75 | — |

The end-point is fairly sharp but the blue colour of the starch iodine quickly reappears. A further experiment was carried out to determine how long it is necessary to allow the mixture of citromycetin and alkaline iodine to react to give consistent results. A solution of citromycetin was made having the same composition as that described above. 10 c.c. of this were measured into each of nine flasks, together with a blank containing 10 c.c. of distilled water. Into each of these flasks were then measured in turn 100 c.c. of water, 40 c.c. of N/10 iodine, and 50 c.c. of N/10 sodium hydroxide

added drop by drop during the space of three minutes. The flasks were now kept at 24° C. for varying lengths of time as indicated in the following table :—

TABLE VIII.—Effect of time on the reaction between citromycetin and alkaline iodine.

| Flask No. | Duration of Oxidation with Alkaline Iodine. | Thio. Reading c.c. N/10. | c.c. N/10 Iodine absorbed per 10 c.c. Citro-mycetin Solution. |
|-----------|---|--------------------------|---|
| | Min. | | |
| 1 | 30 | 33·14 | 6·68 |
| 2 | 86 | 32·61 | 7·21 |
| 3 | 120 | 32·70 | 7·12 |
| 4 | 150 | 32·58 | 7·24 |
| 5 | 180 | 32·54 | 7·28 |
| 6 | 255 | 32·30 | 7·52 |
| | Hrs. | | |
| 7 | 21 | 31·90 | 7·92 |
| 8 | 23 | 31·90 | 7·92 |
| 9 | 69½ | 31·41 | 8·41 |
| Blank | 21½ | 39·82 | — |

It appears from these results that while there is a slight increase in the amount of iodine absorbed if the oxidation is continued for a very long time a fairly steady figure is reached after 1½ to 2 hours, and in subsequent work a 2-hours' oxidation was used in all estimations.

The results are not so good as those obtained with kojic acid, but seemed to support the opinion that one molecule of citromycetin (molecular weight 290) absorbs eight atoms of iodine, and that this may be used as a roughly quantitative method for the estimation of citromycetin in solution.

The following routine method was adopted in estimating citromycetin in metabolism solutions. The sum of the glucose and citromycetin present is found by estimating the total iodine absorbed on treating a portion of the solution with alkaline iodine. Glucose alone is estimated by polarising a portion of the decolorised metabolism solution. The difference between these two figures gives a roughly quantitative idea of the amount of citromycetin present. The best method of decolorising the highly coloured metabolism solutions for polarising was found to be treatment with colloidal iron.

25 c.c. of the metabolism solution are neutralised to phenolphthalein with sodium hydroxide in a 50 c.c. measuring flask. 10 c.c. of colloidal iron are added drop by drop, followed by a little magnesium sulphate solution, and the whole made up to the mark. The mixture is filtered through a dry paper and, if it is still coloured, this residual colour may be discharged by adding one drop of 50 per cent. sulphuric acid. The solution is then polarised as usual. Comparative experiments carried out with colloidal iron,

Merck's blood charcoal, Norite and basic lead acetate showed that while all give satisfactory decolorisation, the use of any of them except colloidal iron is rendered inadvisable because of the large loss of glucose by adsorption.

The effect of alkaline iodine solution on the following derivatives of citromycetin has also been investigated :—

- (a) Citromycin.
- (b) *O*-Dimethylcitromycetin.
- (c) *O*-Dimethylcitromycetin methyl ester.

The results showed that while citromycin is readily oxidized by alkaline iodine, absorbing approximately eight atoms of iodine per molecule of citromycin, in a similar manner to citromycetin itself, neither *O*-dimethylcitromycetin nor its methyl ester are appreciably attacked by alkaline iodine. The details of the experiment with citromycin follow :—

0.2460 gm. (M/100) of citromycin was dissolved in the minimum amount of N/10 sodium hydroxide and made up to 100 c.c. Different amounts of this solution were treated with alkaline iodine in a similar manner to that described for citromycetin. The oxidation was continued for two hours before acidifying and titrating the excess of iodine. The results are given in Table IX.

TABLE IX.—Reaction between citromycin and alkaline iodine.

| Cubic centimetres Citromycin Solution used. | Cubic centimetres Water added. | Cubic centimetres N/10 Iodine. | Cubic centimetres N/10 NaOH. | Cubic centimetres N/10 Thiosulphate. | Cubic centimetres N/10 Iodine absorbed per 10 c.c. Citromycin Solution. |
|---|--------------------------------|--------------------------------|------------------------------|--------------------------------------|---|
| 2 | 98 | 40 | 50 | 37.75 | 9.30 |
| 5 | 95 | 40 | 50 | 35.22 | 8.78 |
| 10 | 90 | 40 | 50 | 31.28 | 8.33 |
| 15 | 85 | 40 | 50 | 27.05 | 8.37 |
| 20 | 80 | 40 | 50 | 22.99 | 8.31 |
| 15 | 35 | 20 | 25 | 7.84 | 7.98 |
| 20 | 30 | 20 | 25 | 4.64 | 7.58 |
| Blank | 100 | 40 | 50 | 39.61 | — |

An attempt was made to isolate the breakdown products resulting from the oxidation of citromycetin by alkaline iodine but did not meet with great success. 3.9 gm. of citromycetin were dissolved in the theoretical amount of barium hydroxide solution and diluted to 7,400 c.c., iodine equivalent to eight atoms was added and the whole shaken until the iodine had dissolved. At the end of three hours the solution smelt strongly of iodoform and contained a yellowish precipitate. This was filtered off and recrystallised from acetone in shining yellow hexagonal plates having a melting point of 122° C.

It was evidently iodoform. In spite of repeated attempts the only other oxidation products identified with certainty were oxalic and acetic acids and carbon dioxide. For this reason the attempt to obtain information as to the constitution of citromycetin by a study of its oxidation products with alkaline iodine was abandoned.

Section 3.—By hydrolysis with alkali.

When citromycetin was subjected to the action of alkali in the presence of air it immediately became dark brown to black in colour, and all attempts to isolate end products by potash fusion of citromycetin in air failed to give any product. For this reason the alkaline hydrolysis of citromycetin was studied in the following manner: 30 gm. of citromycetin were introduced into a hydrolysis flask fitted with a reflux condenser. The whole apparatus was then filled with nitrogen. A solution of 100 gm. of potassium hydroxide in 200 c.c. of water was now added, and the mixture was boiled in an atmosphere of nitrogen for three hours. Air was rigorously excluded during the whole hydrolysis. Even so the hydrolysis solution became very dark brown in colour. The water in the reflux condenser was kept warm and the constant stream of nitrogen bubbled through the apparatus was subsequently scrubbed in ice-cold water. At the end of the hydrolysis this aqueous solution gave strong positive tests for acetone. The solution smelt strongly of acetone, gave a very intense sodium nitroprusside test, and gave a copious precipitate with 2:4-dinitrophenylhydrazine, which on recrystallising from alcohol had a melting point of 126°–127° C. (The melting point of acetone 2:4-dinitrophenylhydrazone is 128° C.)

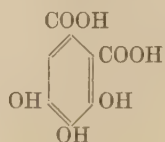
Carbon dioxide was now passed through the hydrolysis mixture to saturation point, contact with air still being avoided. Nothing separated and the liquid retained its dark colour. It was extracted exhaustively with ether, and the ether extract on evaporation gave rise to a very small amount of dark brown material from which nothing crystalline could be isolated. The ether-extracted solution was then acidified with sulphuric acid and the acidified solution extracted exhaustively with ether. The ether solution, on evaporation, yielded large quantities of acetic acid, and, since pure ether was used for extraction, acetic acid must be a product of hydrolysis. The ether extract was dried *in vacuo* over potassium hydroxide and sulphuric acid, giving rise to a brownish black residue containing an obvious white crystalline material. The residue, freed from acetic acid by drying, was then treated with water and warmed for some time on the water bath. This treatment dissolved the dark brown material and left a small amount of insoluble residue in the form of minute rosettes. These were filtered off while still warm, and recrystallised by dissolving in hot alcohol, decolorising with a little charcoal and adding boiling water to the alcoholic solution until crystallisation started. By this means a small quantity of white needles was obtained having the following characteristics:—

They were almost insoluble in water, but readily soluble in hot alcohol. On heating, no change was obvious until 260°–270° C. at which temperature the material became

brown in colour. The colour slowly darkened as the temperature rose until the material finally melted at 290° – 291° C. and decomposed sharply at 292.5° C., with gas evolution, leaving a brownish black residue. An aqueous solution of this material gave with a very small amount of ferric chloride a pure blue colour changing to green on the addition of further amounts of ferric chloride. An alcoholic solution gave a very dark ivy green colour with ferric chloride.

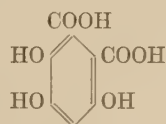
The yield of this material was so very small, however, that there was not sufficient for analysis, but, in view of later results and the characteristics described above, it seems probable that it was a trihydroxyphthalic acid.

Only one trihydroxyphthalic acid has been described, although two are possible, having the two following formulæ:—



(a)

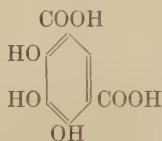
3 : 4 : 5-trihydroxyphthalic acid.



(b)

3 : 5 : 6-trihydroxyphthalic acid.

3 : 4 : 5-trihydroxyphthalic acid was prepared by PRATT and PERKINS (1918). It is described by them as being insoluble in boiling xylene, and on crystallising from water it gave slightly brownish crystals, which melted slowly, with decomposition, beginning at about 280° C. (corr.). It gave a greenish blue colour with a little ferric chloride and a yellow green with excess. PRATT and PERKINS considered their 3 : 4 : 5-trihydroxyphthalic acid to be identical with the so-called gallocarbonic acid of SENHOFER and BRUNNER (1880). Gallocarbonic acid has a melting point of over 270° C. with evolution of carbon dioxide. With very dilute ferric chloride it gives a violet colour, with more concentrated, a greenish brown colour. As the result of more recent work, however, by VOSWINCKEL and DE WEERTH (1912), and FEIST and SANDSTEDT (1918) it seems certain that gallocarbonic acid is not a phthalic acid derivative but an *iso*-phthalic acid derivative of the following formula:—



In view of these facts, together with considerations arising from the results obtained by the hydrolysis of *O*-dimethylcitromycetin methyl ester (see p. 237), it is probable that the trihydroxyphthalic acid isolated from the hydrolytic products of citromycetin is represented by formula (b) and is indeed 3 : 5 : 6-trihydroxyphthalic acid.

A further experiment was carried out with a view to estimating the volatile materials produced by alkaline hydrolysis of citromycetin. 3.38 gm. of pure citromycetin were

introduced into a 100 c.c. Claisen distilling flask connected to a condenser and an adapter, the end of which passed under a little water contained in a 200 c.c. measuring flask immersed in ice. Nitrogen was bubbled through the whole apparatus until the latter was free from air, and then a solution of 10 gm. of potassium hydroxide in 20 c.c. of water was added through a tap funnel and hydrolysis carried out for two hours. The liquid which slowly distilled over from the Claisen flask was replaced by adding water from time to time through the tap funnel. The distillate A was made up to 200 c.c. and the residue B treated as described later.

Treatment of Distillate A.—An estimation by MESSINGER's method of the acetone present in a portion of the distillate gave 0.223 gm. corresponding to 0.37 gm. molecules per gm. molecule of citromycetin. The 2:4-dinitrophenylhydrazone was prepared and recrystallised. It melted at 127°–128° C., and its melting point was unchanged on admixture with acetone 2:4-dinitrophenylhydrazone prepared from a sample of pure acetone. On the other hand, a sample of the 2:4-dinitrophenylhydrazone of methyl ethyl ketone, with which acetone easily might be confused, was found to melt at 114°–115° C. while the mixed melting point of this with the hydrazone isolated from the hydrolysis mixture proved to be 94°–99° C. On combustion 2.969 mgm. of the hydrazone of the hydrolysis product gave 0.597 c.c. nitrogen at 23.5° C. and 762 mm. pressure (SCHOELLER, Berlin), corresponding to 23.2 per cent. of nitrogen (theoretical for acetone 2:4-dinitrophenylhydrazone = 23.5 per cent., and for methyl ethyl ketone 2:4-dinitrophenylhydrazone = 22.2 per cent.).

Treatment of Residue B.—The condenser and adapter were now detached from the Claisen distilling flask which was then connected to a large bubbler containing standard barium hydroxide. A slight excess of acid was run through the tap funnel, and the CO₂ liberated was passed over in a current of nitrogen, absorbed and estimated. 0.54 gm. CO₂ was produced corresponding to 1.09 gm. mol. CO₂ per gm. mol. of citromycetin.

The acidified hydrolysis mixture was now exhaustively evaporated *in vacuo* to remove all volatile acids and the combined distillates titrated with N/10 sodium hydroxide, of which 75.1 c.c. were needed to neutralise, corresponding to 0.72 gm. mol. volatile acid per gm. mol. citromycetin. The volatile acid was shown to be acetic acid by conversion into the silver salt. 0.0788 gm. of the silver salt gave 0.0506 gm. of silver, corresponding to 64.23 per cent. Ag (theoretical for silver acetate = 64.64 per cent.).

The volatile hydrolysis products per gm. mol. of citromycetin are thus:—0.37 gm. mol. acetone, 1.09 gm. mol. carbon dioxide and 0.72 gm. mol. acetic acid.

In a repeat experiment the volatile hydrolysis products obtained per gm. mol. of citromycetin were 0.36 gm. mol. acetone, 1.14 gm. mol. carbon dioxide, and 0.92 gm. mol. acetic acid. The volatile acid was examined specially for formic acid but none was found.

SECTION 4.—*Hydrolysis of the methyl ester of O-dimethylcitromycetin with alcoholic potassium hydroxide.*

In view of the failures so far experienced in attempting to isolate decomposition products from the hydrolytic products from citromycetin itself, all subsequent work was devoted to the examination of the products of hydrolysis with alcoholic potassium hydroxide of the methyl ester of *O*-dimethylcitromycetin. In this way, by replacing OH groups with methoxyl groups before hydrolysis, most of the trouble due to the formation of black decomposition products was overcome and it was found possible to isolate various decomposition products. 30 gm. of the methyl ester of *O*-dimethylcitromycetin were boiled with alcoholic potassium hydroxide made from 60 gm. of potassium hydroxide sticks + 60 c.c. of water + 240 c.c. of absolute alcohol. The methylated citromycetin was placed in a hydrolysis flask fitted with reflux condenser. A stream of nitrogen free from oxygen was bubbled through this, and when all the air had been driven out, the alcoholic potassium hydroxide was run in through a tap funnel placed at the top of the condenser. The contents were boiled for $3\frac{1}{2}$ hours, and at the end of this period the alcohol was completely removed *in vacuo*, the residue taken up in 800–900 c.c. of distilled water and carbon dioxide passed in to saturation. The hydrolysis mixture which, previous to passing in carbon dioxide, was dark brown in colour was now much lighter and was thoroughly extracted with ether. The ether extract on evaporation gave Fraction A, treatment of which is described below. The ether-extracted solution was acidified with sulphuric acid, giving rise to a precipitate which was filtered off, and the filtered acid solution extracted with ether. This ether extract gave Fraction B.

Treatment of Fraction A.—Fraction A consisted of a yellow crystalline solid (Product A). It was purified by crystallising from boiling absolute alcohol from which it separated in light yellow, very fine, feathery, needles having a melting point of 177° – 180° C. and showing signs of decomposition on melting. An alcoholic solution of this substance gave an olive brown colour with ferric chloride.

On the addition of alcoholic potassium acetate solution to a solution of Product A in absolute alcohol a potassium salt was formed. This separated at first in a gelatinous condition, but on standing gradually crystallised in thick rosettes of orange prisms. Product A also gave a hydrobromide, crystallising in bunches of orange needles, on the addition of concentrated hydrobromic acid to its solution in glacial acetic acid. In both cases there was immediate darkening in colour from yellow to orange, on the addition of potassium acetate in the one case, and of hydrobromic acid in the other. A darker colour was obtained with hydrobromic acid than with potassium acetate. These properties indicate that the pyrone ring originally present in citromycetin still remains unbroken in Product A.

It did not contain water of crystallisation and could be dried unchanged at 110° C. in nitrogen, although it showed some signs of decomposition at 150° C. in nitrogen. It

had a molecular weight, determined by BARGER'S method, of between 231 and 250 (molecular weight of $C_{12}H_{12}O_5 = 236$). ZEISEL estimations on material dried at $110^\circ C$. in nitrogen gave the following results: 0.1664 gm. of substance gave 0.3412 gm. of AgI and 0.1904 gm. substance gave 0.3872 gm. AgI corresponding to 27.06 per cent. OCH_3 and 26.86 per cent. OCH_3 respectively. (Theoretical for $C_{10}H_6O_3 \cdot (OCH_3)_2$, *i.e.*, $C_{12}H_{12}O_5 = 26.28$ per cent.) The following combustion results were obtained on different samples dried in nitrogen at $110^\circ C$:—

| Weight of Substance Analysed. | Weight of CO_2 . | Weight of H_2O . | Percentage Carbon. | Percentage Hydrogen. |
|-----------------------------------|-----------------------|-----------------------|-----------------------|-------------------------|
| 0.1212 gm. | 0.2717 | 0.0589 | 61.14 | 5.44 |
| 0.1194 gm. | 0.2664 | 0.0569 | 60.87 | 5.33 |
| Theoretical for $C_{12}H_{12}O_5$ | — | — | 61.02 | 5.10 |

Product A appears to be a dimethoxy-hydroxy-methylbenzopyrone.

Treatment of Fraction B.—This fraction was the main hydrolytic product of the methyl ester, 8.2 gm. being obtained from 30 gm. of ester. It consisted of a light brown syrup smelling strongly of acetic acid, which is thus one of the hydrolytic products of the methyl ester. It gave a brownish purple colour with ferric chloride. On standing it gradually set to a thick sticky mass of needles which could not be purified by draining on a porous tile. It was finally fractionated in the following manner: it was dried *in vacuo* over potassium hydroxide to remove acetic acid, and the residue dissolved in 200 c.c. of hot absolute alcohol, filtered and heated to boiling. To the boiling solution were added 80 c.c. of 5 per cent. alcoholic solution of potassium acetate. On cooling there was a copious separation of a potassium salt which, however, did not crystallise but appeared under the microscope as small spherical globules. These were filtered off and washed and constituted Fraction B1. (Weight = 5.9 gm.)

The filtrate constituting Fraction B2 will be dealt with later.

The potassium salt (Fraction B1) was dissolved in water and acidified with a little 50 per cent. sulphuric acid. This produced an immediate milkiness which, on shaking vigorously, coalesced into a single piece of brown, tarry material. The clear aqueous solution, which was only pale yellowish brown in colour, was extracted with ether, and the ether solution evaporated to dryness, leaving a transparent non-crystalline residue which was now fractionated from a considerable volume of dry chloroform. The main product separating from a hot chloroform solution on cooling consisted of a substance crystallising in white needles, an aqueous solution of which gave a pure purple colour with ferric chloride (Product B). It was readily soluble in water and alcohol and could be crystallised by dissolving in a large volume of chloroform and evaporating the solution quickly until crystals appeared. On heating it melted with decomposition at 181° – $182^\circ C$, but the melting point varied very considerably with the rate of heating.

The crystals clung tenaciously to chloroform which could only be driven off by heating to constant weight at 110°C . in nitrogen, when they lost about 14 per cent. of their weight as chloroform.

This substance on analysis gave the following results :—

0.2389 gm. dried to constant weight at 110°C . in nitrogen was titrated with N/10 sodium hydroxide to phenolphthalein: A very sharp end-point was obtained, and 19.45 c.c of N/10 sodium hydroxide solution were required, corresponding to a combining weight of 122.8 and a molecular weight of 122.8 or 245.6. (Theoretical for $\text{C}_{10}\text{H}_{10}\text{O}_7 = 242$.)

ZEISEL estimations gave the following results: 0.1210 gm. of dried material gave 0.2308 gm. of AgI and 0.1248 gm. gave 0.2376 gm. of AgI, corresponding to 25.19 per cent. and 25.14 per cent. OCH_3 respectively. (Theoretical for $\text{C}_{10}\text{H}_{10}\text{O}_7 = \text{C}_8\text{H}_4\text{O}_5 \cdot (\text{OCH}_3)_2 = 25.63$ per cent. OCH_3 .)

It gave the following results on combustion :—

| Weight of Substance. | Weight of CO_2 . | Weight of H_2O . | Percentage Carbon. | Percentage Hydrogen. |
|--|---------------------------|----------------------------------|--------------------|----------------------|
| 0.1122 gm. | 0.2068 | 0.0405 | 50.27 | 4.04 |
| 0.1357 gm. | 0.2482 | 0.0496 | 49.89 | 4.09 |
| Theoretical for $\text{C}_{10}\text{H}_{10}\text{O}_7$ | — | — | 49.58 | 4.17 |

The substance appears to be a dimethoxy-hydroxy-phthalic acid.

That it is a true phthalic acid derivative and not an *iso*- or *tere*-phthalic acid derivative seems to be indicated by the following fact: A small amount of the dimethoxy-hydroxy-phthalic acid was heated in an open test tube in a castor oil bath, the temperature of which was maintained at 180° – 200°C . Water was given off, as evidenced by the condensation of moisture on the cool parts of the tube. A substance sublimed (Product C), with apparent gaseous decomposition, in the form of beautiful long white needles some of which were half a centimetre long. These melted at 203° – 204°C . without decomposition but with partial sublimation during heating. It was readily soluble in water but gave no colour with aqueous ferric chloride.

Product B was definitely proved to be a true phthalic acid derivative as follows: A quantity of the dry material was dissolved in dry ether and ethylated by treatment with an excess of an ethereal solution of diazoethane. On removal of the excess diazoethane and ether an oil remained which no longer gave a colour with ferric chloride. This oil was hydrolysed by heating on the boiling water bath with aqueous potassium hydroxide solution and the hydrolysis solution was acidified and extracted with ether. On evaporating the ethereal solution a crystalline residue remained which was repeatedly sublimed in an oil bath at 150° – 200°C . at a pressure of 14 mm. until a constant melting point was obtained. It was finally sublimed at ordinary pressure and analysed.

The final product (Product D) consisted of beautiful slender prisms of a very pale lemon-yellow colour, which melted sharply at 195°–196° C. It is insoluble in water, cold alcohol, ligroin and petroleum ether. It is soluble in acetone, chloroform, benzene and is readily crystallised from hot alcohol or aqueous alcohol.

The sublimed product (Product D) was proved by analysis (SCHOELLER, Berlin) to be dimethoxy-ethoxy-phthalicanhydride, $C_6H \cdot (OCH_3)_2 \cdot (OC_2H_5) \begin{matrix} \diagup CO \\ \diagdown CO \end{matrix} O = C_{12}H_{12}O_6$ and not dimethoxy-ethoxyphthalic acid, $C_{12}H_{14}O_7$.

4.289 mgm. gave 8.990 mgm. CO_2 and 1.90 mgm. H_2O corresponding to 57.18 per cent. carbon and 4.92 per cent. hydrogen. (Theoretical for $C_{12}H_{12}O_6 = 57.15$ per cent. carbon and 4.76 per cent. hydrogen; for $C_{12}H_{14}O_7 = 53.31$ per cent. carbon and 5.22 per cent. hydrogen.)

2.758 mgm. gave 7.640 mgm. AgI.

(Theoretical for $C_6H \cdot (OCH_3)_2 \cdot (OC_2H_5) \begin{matrix} \diagup CO \\ \diagdown CO \end{matrix} O = 7.680$ mgm. AgI; for

$C_6H \cdot (OCH_3)_2 \cdot (OC_2H_5) \begin{matrix} \diagup COOH \\ \diagdown COOH \end{matrix} = 7.170$ mgm. AgI.)

Product D is thus the anhydride and not the free acid and hence it follows that the substance from which it arose, *i.e.*, Product B, is a true phthalic acid derivative and not an *iso*- or a *tere*-phthalic acid derivative.

In view of the lack of published information on the dimethoxy-hydroxy-phthalic and benzoic acids an attempt was made to prepare the parent phenolic acid from the residual products of the treatment of the dimethoxy-hydroxy-phthalic acid with hydriodic acid as carried out in the ZEISEL estimation. 1.87 gm. of the dimethoxy-hydroxy-phthalic acid, isolated as described on p. 238, were boiled with 15 c.c. of freshly distilled, constant boiling point, hydriodic acid in an atmosphere of carbon dioxide. The hydriodic acid solution was cooled, diluted with four times its volume of water and extracted with ether. The ether extract was washed with water to remove hydriodic acid and with sodium thiosulphate solution to remove iodine. The purified ether solution on evaporation to dryness left a crystalline residue (Product E) which was crystallised from a large volume of chloroform, from which it separated in white prisms having a melting point which varied considerably with the rate of heating. The substance melted in any case with decomposition, but if heated slowly had a melting point of 208°–209° C., while if heated quickly it melted at 214°–215° C. An aqueous solution of the material gave a deep blue colour with ferric chloride, fading to brown with excess of reagent. An aqueous solution immediately reduced ammoniacal silver solution in the cold, and aqueous silver nitrate solution, also in the cold, after standing for a short time.

A portion of this material was heated in a castor oil bath at a temperature somewhat above its melting point. The tube containing the material was fitted with a short inner tube kept cold by a current of cold water. After some time there appeared on the inner tube a sublimate consisting of a very light white deposit of fine needles (Product F). These were readily soluble in water, giving a colourless solution which quickly turned pink and later brown in colour. On heating, the crystals shrank at $139^{\circ}\text{C}.$ and melted sharply at $140.5^{\circ}\text{C}.$ without decomposition.

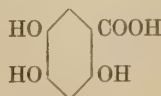
An aqueous solution of the material gave a very transient blue colour with ferric chloride.

Treatment of Fraction B2.—The alcoholic filtrate constituting Fraction B2 was evaporated to dryness *in vacuo*, the residue dissolved in water and acidified with sulphuric acid. A resinous precipitate which formed was filtered off and the sulphuric acid solution was extracted with ether. The ether residue was taken up with chloroform, which dissolved the greater part, and from the chloroform solution was obtained a further amount of dimethoxy-hydroxy-phthalic acid as described on p. 238, with a melting point of 181° – $182^{\circ}\text{C}.$ The insoluble residue, consisting of fine needles, was crystallised from 50 per cent. aqueous alcohol, from which it separated in small white needles which were apparently only very slightly soluble either in water or 50 per cent. alcohol. These had a melting point of 242° – $243^{\circ}\text{C}.$ with decomposition, and gave no colour with ferric chloride either in alcoholic or aqueous solution. This fraction, which was small in amount, has not been further investigated. It appeared to be much less readily extracted from the acidified hydrolysis mixture than was dimethoxy-hydroxy-phthalic acid since, while the latter was almost completely extracted by two extractions with ether, the former was still being extracted in small amounts after six or seven extractions.

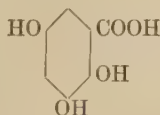
Discussion of results obtained and their bearing on the constitution of citromyccetin.

Product F is almost certainly hydroxyquinol. A sample of hydroxyquinol made by THIELE'S method (1898) from benzoquinone shrinks at $139^{\circ}\text{C}.$ and melts at 140.5° – $141^{\circ}\text{C}.$ There is no obvious change in melting point of a mixture of the synthetic material with Product F.

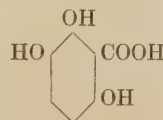
Product E is therefore a hydroxyquinolcarboxylic acid. There are three possible isomers of this compound.



(a)



(b)

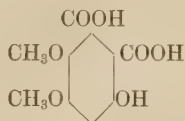


(c)

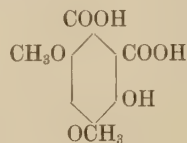
Of these only (a) has been described and this is apparently identical with Product E. The hydroxyquinolcarboxylic acid of the formula (a) was first prepared by THIELE and JAEGER (1901), by heating together hydroxyquinol and sodium bicarbonate in water. Its constitution was considered by VON HEMMELMAYR (1911) to be represented by formula (c) but it was finally settled by BERGELLINI and MARTEGIANI (1912), who showed that, since on methylation with methyl sulphate it gives asaronic acid, it must have the constitution given in formula (a). A specimen of synthetic 1:3:4-trihydroxybenzoic acid (a) was prepared by THIELE and JAEGER'S method given above and was shown to behave similarly to the product isolated from citromycetin. The variation in melting point with the rate of heating makes a mixed melting point of less diagnostic value than usual, but the melting point of a mixture of synthetic 1:3:4-trihydroxybenzoic acid and Product E was the same as Product E itself when the two melting points were determined side by side in the same apparatus.

Accepting that Product E is 1:3:4-trihydroxybenzoic acid then Product B, dimethoxy-hydroxy-phthalic acid, must of necessity have one of the two following formulæ since:—

- (a) It is a true phthalic acid and not an *iso*- or a *tere*-phthalic acid, and hence has its carboxyl groups vicinal to each other and
- (b) the free hydroxyl group must be in the *ortho* position to one of the carboxyl groups since the material arises from fully methylated citromycetin, which is a pyrone derivative.

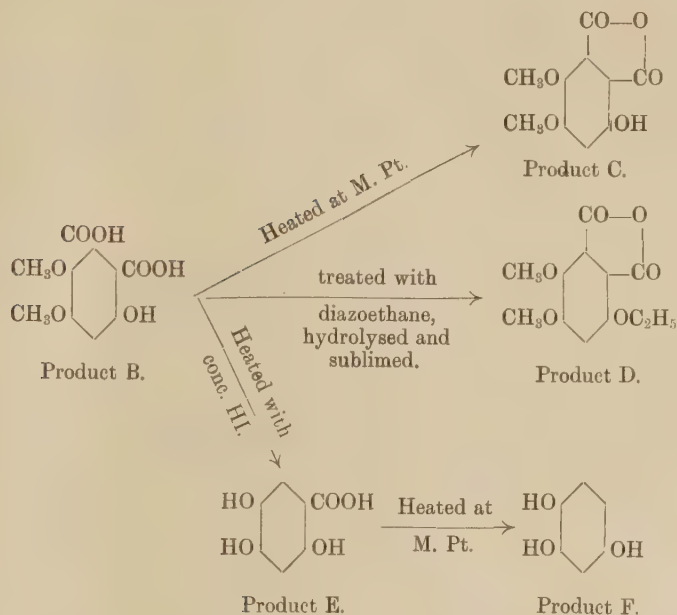


(1)

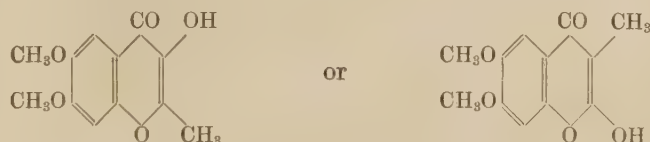


(2)

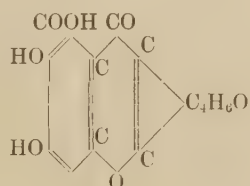
It has not yet been definitely established which of these formulæ is correct but formula (1) seems the more probable because of the strong dyeing properties of citromycetin (p. 244). It has been shown by LIEBERMANN and V. KOSTANECKI that marked dyeing properties in the natural colouring matters are almost invariably associated with the presence in the molecule of two hydroxyl groups in the *ortho* position to one another. Hence formula (1) may be taken as representing the dimethoxy-hydroxy-phthalic acid, and accepting this, the relationship of the various breakdown products arising from methylated citromycetin is shown in the following scheme:—



The formula for Product A then becomes one of the two following:—



and the formula for citromycetin becomes



The question of the seventh oxygen atom present in the group $\text{C}_4\text{H}_6\text{O}$ has not been satisfactorily settled, and work is at present in progress to settle the constitution of this group. It is of interest in this connection, however, to note that alkaline hydrolysis of citromycetin or of its fully methylated derivative gives rise to considerable quantities of acetone, together with acetic acid and carbon dioxide (p. 236).

Dyeing properties of citromycetin.

A sample of citromycetin was submitted to Prof. A. G. PERKIN of the Department of Colour Chemistry and Dyeing, Leeds University, with the request that Prof. PERKIN should give an opinion as to whether citromycetin has any marked dyeing properties. The following reply was received from him, for which we desire to offer him our thanks.

“ 29th October, 1926.

“ I have made a cursory examination of your sample of citromycetin and find it to be quite a strong dyestuff. The shades given closely resemble those of luteolin, the colouring matter of WELD, though your product is not the same as this.

“ The reactions of the compound correspond to some extent with dyes of the xanthone or flavone group, more nearly perhaps to the former, in that it yields a green fluorescent solution with sulphuric acid.”

Summary.

The production of a new biochemical product—citromycetin—($C_{14}H_{10}O_7 \cdot 2H_2O$) by various species of *Citromyces* is described. The influence of different factors on the yield of this material is given on pp. 212–215. Details of the method of preparation of citromycetin on a large laboratory scale are given on pp. 215–217. The general properties of the substance are described on pp. 218–221. The preparation and properties of a number of derivatives of citromycetin and of its decarboxylated product citromycin are given on pp. 221–230. The decomposition products of citromycetin are described (*a*) by acid hydrolysis (p. 230), (*b*) with alkaline iodine (p. 231), (*c*) by alkaline hydrolysis (p. 234), (*d*) by hydrolysis of methylated citromycetin with alcoholic potassium hydroxide (p. 237). The question of the constitution of citromycetin is discussed on pp. 241–243. A report on the dyeing properties of citromycetin, submitted by Prof. A. G. PERKIN, is given on p. 244.

Studies in the Biochemistry of Micro-organisms.

PART XII.—*On a new methoxy-dihydroxy-toluquinone produced from glucose by species of Penicillium of the P. spinulosum series.*

By JOHN HOWARD BIRKINSHAW and HAROLD RAISTRICK.

Reference to the carbon balance sheets given in Part IV for species of *Penicillium* shows that three species of *Penicillium*, diagnosed by Dr. CHARLES THOM as belonging to the *P. spinulosum* series, were included in Table I. The only salient feature of the carbon balance sheets of these three species is the large amount of acid, entirely of the non-volatile type, which they produce from glucose.

These three species—Ad. 74, Ad. 79 and Ad. 80—show, in addition, a very characteristic colour reaction when grown on a modified CZAPEK-DOX glucose medium containing twenty times the usual amount of ferrous sulphate given in Part I, p. 7.

When the above moulds were grown on this medium at 23° C. it was found that a purple ring first appeared immediately under the mycelium and gradually spread downwards till the whole medium became purple. In later stages of growth a yellow ring was formed below the mycelium and it also spread downwards till the purple colour had almost entirely disappeared. When the slightly yellow medium was exposed to the air, as for instance in filtration of the medium, the liquid was found to assume a much darker tint, almost comparable with the original purple.

On addition of ferric chloride solution to the filtered metabolism solution a characteristic deep brown colour was produced. Addition of dilute permanganate solution, in the cold, to the filtered metabolism solution resulted in an immediate reduction of the permanganate, the amount of reduction being roughly proportional to the depth of the purple colour and to the intensity of the ferric chloride reaction.

The work described in this paper is divided into two parts. The first part deals with the isolation and identification of the acid—citric acid—which is largely responsible for the abnormal acidity produced by this group of fungi. The second part deals with the isolation, properties and constitution of the compound responsible for the characteristic colour of the medium and for the ferric chloride and permanganate reactions. This substance, which is itself a strong acid and contributes in part to the acidity of the medium, is a methoxy-dihydroxy-toluquinone, crystallising in purple-black crystals similar in appearance to potassium permanganate.

PART I.—*Investigation of the nature of the acids produced from glucose by Ad. 74, Ad. 79 and Ad. 80.*

The salient features of the carbon balance sheets for the *Penicillium* species Ad. 74, Ad. 79 and Ad. 80, given on p. 56 of Part IV, are the following :—

- (a) Titratable acidity = 29·1 c.c., 20·3 c.c., 21·7 c.c. N/1 acid per 250 c.c. medium.
- (b) Carbon in non-volatile acids = 0·937 gm., 0·862 gm., 0·799 gm., corresponding respectively to 20·0 per cent., 18·3 per cent. and 22·6 per cent. of the glucose fermented.

The investigation of the nature of the acid chiefly responsible for these figures was carried out as follows :—

The mould Ad. 74 was grown in a series of 43 one litre conical flasks, plugged with cotton wool, each containing 350 c.c. of CZAPEK-DOX glucose medium, and incubated for 18 days at 24° C. The volume of the filtered metabolism solution was 13·8 litres. To this was added a small amount of washings from the mycelium.

The free acids were neutralised with sodium hydroxide and the solution was precipitated with basic lead acetate. The lead precipitate was well washed and then ground up with an excess of dilute sulphuric acid. The lead sulphate was filtered, the excess sulphuric acid was quantitatively removed from the filtrate with baryta and the filtrate evaporated *in vacuo* down to a syrup. This was twice taken up in methyl alcohol and re-evaporated so as to remove as much water as possible. The residue was finally treated with 400 c.c. of dry methyl alcohol and then saturated with dry hydrochloric acid gas. Crystals separated overnight and were filtered off, washed with methyl alcohol and dried at 60° C. The washings were mixed with the main portion of the filtrate and the passage of the hydrochloric acid gas continued. No further crystals were obtained, so the alcoholic solution was evaporated down to 100 c.c. and re-saturated with hydrochloric acid gas. A further crop of crystals obtained was filtered off and washed. Weight of 1st crop of crystals = 58 gm. Weight of 2nd crop of crystals = 10 gm.

10 gm. of the first crop of crystals were recrystallised from water. They now melted at 76°–78° C., which agrees with trimethyl citrate (M. Pt. 78·5° C.). For confirmation, 5 gm. of the above methyl ester and 25 c.c. of freshly saturated aqueous ammonia solution were mixed and allowed to stand overnight. A fair crop of crystals separated—2·6 gm.—which when recrystallised from water melted at 206°–207° C. Citramide from pure citric acid melted at 207°–208° C. The mixed melting point was 207°–208° C. For further confirmation 0·5 gm. of the amide from Ad. 74 was heated with 2 c.c. of 72 per cent. sulphuric acid in a test tube to 130° C. and poured into water. A yellow crystalline precipitate appeared, which was filtered and washed with water. This gave the characteristic reactions of citrazinic acid—blue fluorescence with ammonia, and a deep blue colour when dropped into boiling sodium nitrite solution. All the

above tests serve to identify completely the main acid product of Ad. 74 as citric acid.

The second crop of crystals, when recrystallised from water with the addition of a little charcoal, melted at 76°–78° C. and evidently also consisted of trimethyl citrate.

The alcoholic filtrate from the crystals was extracted with ether, after removal of alcohol *in vacuo*, and the extract was washed with dilute potassium hydroxide solution and then with a little water. After drying over anhydrous sodium sulphate the ether was distilled and the residue poured into a tube. It immediately began to crystallise and soon set solid. Weight = 1.89 gm. After recrystallisation from water it melted at 74°–76° C. and proved to be trimethyl citrate. Thus, the only esterifiable acid formed by Ad. 74 under the conditions adopted is citric acid.

The acids from Ad. 79 and Ad. 80 were also investigated and were found to consist chiefly of citric acid.

In the case of Ad. 80 the ethyl esters were prepared and the portion insoluble in ether appeared to be considerable. An attempt was made to distil it in a high vacuum using a mercury vapour pump, but this met with no success.

To sum up then, the acid which is mainly responsible for the high acidity and high "carbon in non-volatile acids" in each of the three species of *Penicillium*—Ad. 74, Ad. 79, Ad. 80—is citric acid.

PART 2.—*Preparation, properties and constitution of the substance produced by Ad. 74, Ad. 79 and Ad. 80, which gives the characteristic colour to the medium and the reactions with ferric chloride and permanganate.*

N.B.—For convenience, and in the absence of a name, this substance will be referred to briefly as "Z."

Preparation of Z.—Sixty litres of the CZAPEK-DOX glucose solution rich in iron was distributed between 12 trays and placed in the combined sterilizer-incubator used for the preparation of kojic acid and described on p. 136 (Part VII). After sterilization each tray was sown with an emulsion of spores of culture X. 80, prepared from a Roux bottle culture on beer wort agar. (X. 80 is another strain of the same group as Ad. 74, Ad. 79 and Ad. 80, isolated at a later date. It was chosen in preference to any of the other strains as it gives a deeper colour reaction. Z has, however, been isolated from each of the species Ad. 74, Ad. 79 and Ad. 80.) The trays were incubated at room temperature—an average of about 22° C.—and an abundant supply of sterile air was passed through the incubator during the whole incubation period. The trays were sampled on two occasions with the following results (See Table I, Tank Expt. Z1.):—

TABLE I.—*Tank Experiment Z 1.*

| Tray No. | 7 Days. | | | | 11 Days. | | | |
|----------|---|---|---------------------------------|--------------------------------|---|---|---------------------------------|--------------------------------|
| | Titration, c.c. N/1 Alkali per 25 c.c. | c.c. N/10 KMnO ₄ per 25 c.c. | Glucose by Polari- meter. | FeCl ₃ Reaction. | Titration, c.c. N/1 Alkali per 25 c.c. | c.c. N/10 KMnO ₄ per 25 c.c. | Glucose by Polari- meter. | FeCl ₃ Reaction. |
| 1 | — | — | Per cent. — | — | 2.40 | 5.0 | Per cent. 0.714 | Strong reaction |
| 2 | 1.35 | 2.8 | 2.55 | + + | 2.20 | 5.8 | 0.953 | " |
| 3 | — | — | — | — | 2.16 | 4.8 | 1.044 | " |
| 4 | — | — | — | — | 2.30 | 6.0 | 0.777 | " |
| 5 | 1.18 | 3.3 | 2.44 | + + + | 2.27 | 7.2 | 0.601 | " |
| 6 | — | — | — | — | 2.44 | 5.8 | 1.158 | " |
| 7 | — | — | — | — | 2.42 | 6.3 | 0.787 | " |
| 8 | 1.55 | 3.1 | 2.48 | + + + | 2.35 | 6.0 | 0.822 | " |
| 9 | — | — | — | — | 2.45 | 4.8 | 0.985 | " |
| 10 | — | — | — | — | 2.70 | 5.3 | 0.855 | " |
| 11 | 1.15 | 2.4 | 2.48 | + + | 2.46 | 5.8 | 1.022 | " |
| 12 | — | — | — | — | 2.52 | 5.0 | 1.061 | " |

After 14 days' incubation the metabolism solution, which was dark purple in colour, was filtered and analysed. 44.13 litres were obtained, having the following characteristics :—

- (1) *Titration*, 22.15 c.c. N/1 NaOH for 250 c.c.
- (2) *KMnO₄*, 5.5 c.c. N/1 KMnO₄ for 250 c.c.
- (3) *Glucose* (by polarimeter), 0.318 per cent.

The whole of this liquid was evaporated *in vacuo* to 1,520 c.c. Several methods for the isolation of Z were tried, but these were not very successful until it was realized that Z is present in solution, partly free, and partly as a complex which on hydrolysis with acids breaks down. The method finally adopted was as follows :—

Sufficient sulphuric acid was added to the evaporated metabolism solution to bring the solution to normality, and the mixture was boiled for several hours under a reflux condenser. The cooled solution was then thoroughly extracted with ether, which extracts all the Z present, together with large amounts of citric acid. A quantity of Z separated from the evaporated ether solution, and the remainder was separated by extraction of the mother liquors, after dissolving in water, with toluene. Most of the toluene was evaporated *in vacuo*, when Z crystallised out. The crude Z, whether from ether or toluene, was finally purified by two sublimations *in vacuo*. Z begins to sublime at 120° C. at about 1 mm., giving a pure product in purple-black crystals.

Properties of Z.—The substance Z forms dark coloured, almost black, crystals with a metallic lustre, having the appearance of brownish plates under the microscope. It

is very slightly soluble in cold water, more soluble in hot water, giving an amethyst shade which on acidifying changes first to pink and then to yellow. The change in colour from purple to yellow on acidification explains the characteristic colour change in the medium observed with all strains of *Penicillium* in this group and previously given in detail. When potassium or sodium hydroxide is added to neutralise its acid reaction the solution becomes deep purple, changing to faint blue when the reaction is made more alkaline. With concentrated sulphuric acid Z gives an intense blue colour.

On heating, Z melts without decomposition at 202° – 203.5° C. and sublimes to some extent on the M. Pt. tube.

Z has a definite aromatic odour, reminiscent of the quinones.

It immediately decolorises acidified permanganate solution in the cold, and gives a deep rich brown colour with ferric chloride.

It is reduced by zinc dust and hydrochloric acid to a colourless compound. This on extraction with ether gave colourless microscopic crystals, but these very quickly darkened owing to atmospheric oxidation. The reduced Z is readily soluble in water giving a colourless solution which, on standing, quickly absorbs oxygen and becomes pink in colour. A freshly prepared solution of reduced Z in water gives an intense blue-green colour with ferric chloride.

A re-sublimed sample of Z gave the following results on combustion:—

| Weight of Z analysed. | Weight of H_2O . | Weight of CO_2 . | Percentage of Hydrogen. | Percentage of Carbon. |
|----------------------------|--------------------|--------------------|-------------------------|-----------------------|
| | Gm. | Gm. | | |
| 0.1759 gm. | 0.0702 | 0.3372 | 4.47 | 52.28 |
| 0.1726 gm. | 0.0661 | 0.3299 | 4.29 | 52.14 |
| Calculated for $C_8H_8O_5$ | — | — | 4.38 | 52.16 |

The empirical formula for Z is therefore $C_8H_8O_5$.

Determination of molecular weight of Z.—(a) A determination of the molecular weight of Z was carried out by RAST's camphor method (1922). 0.0266 gm. of Z mixed with 0.1409 gm. of camphor lowered the melting point of the latter 41° C. This corresponds to a molecular weight of 184. (Theoretical for $C_8H_8O_5 = 184$.)

(b) *By titration with sodium hydroxide.*—0.1141 gm. of Z was weighed out and titrated with N/10 sodium hydroxide to phenolphthalein. In order to ensure complete solution an excess of sodium hydroxide was added and then the solution was titrated back with N/10 hydrochloric acid. Owing to the dark permanganate colour of the solution the end-point was difficult to determine, and the figure obtained can only be regarded as a rough approximation.

22.0 c.c. N/10 NaOH were added and 8.8 c.c. N/10 HCl were required for back titration, giving a net value of 13.2 c.c. N/10 NaOH.

Assuming that the substance is a monobasic acid this corresponds to a combining weight of 86.4. Since the molecular weight of Z has been shown to be 184 it is obvious that Z titrates as a dibasic acid to phenolphthalein.

Preparation of the diacetyl compound of Z, $C_8H_6O_3 (O.CO.CH_3)_2$.—0.5 gm. of Z was mixed with 5 c.c. of acetic anhydride and three drops of concentrated sulphuric acid. The mixture was heated for a few seconds, when the original purple colour had completely disappeared. The mixture was then poured into cold water and crystals quickly separated which were filtered and dried. Yield of crude product = 0.65 gm. of yellow needles. This was recrystallised from absolute alcohol in yellow needles having a melting point of $139.5^\circ C$.

This compound gave the following results on combustion :—

| Weight of Acetyl Compound Analysed. | Weight of H_2O . | Weight of CO_2 . | Percentage of Hydrogen. | Percentage of Carbon. |
|---|--------------------|--------------------|-------------------------|-----------------------|
| 0.1543 gm. | Gm. 0.0624 | Gm. 0.3057 | 4.53 | 54.02 |
| 0.1508 gm. | 0.0592 | 0.2956 | 4.39 | 53.57 |
| Theoretical for $C_8H_6O_3 (O.CO.CH_3)_2$ | — | — | 4.51 | 53.71 |

The number of acetyl groups present was estimated in the following manner :—

0.2705 gm. of the acetyl compound was hydrolysed by heating under a reflux with 25 c.c. of N/1 sulphuric acid in an oil bath at $130^\circ C$. for 7 hours. The mixture was then made up to about 500 c.c. with distilled water, and evaporated *in vacuo* to about 25 c.c., the operation being repeated until a constant titration figure was obtained for the distillate. This was taken as the blank for each distillation and the total corrected titration figure was 20.37 c.c. of N/10 sodium hydroxide.

This corresponds to a CH_3CO content of 32.38 per cent.

Calculated for two acetyl groups in $C_8H_6O_3 (O.CO.CH_3)_2 = 32.10$ per cent. These figures indicate clearly that the compound is a diacetyl derivative, and prove the presence in Z of two acetylatable hydroxyl groups.

During the distillation a very small amount of Z came over and tinged the distillate pink. This would not affect the result, however, as the amount would be practically the same in each distillation and would be included in the blank.

Preparation of the tetra-acetyl compound of reduced Z, $C_8H_6O (O.CO.CH_3)_4$.—As it had previously been shown that the reduction product of Z, formed by treating Z with zinc dust and hydrochloric acid, was easily oxidized by atmospheric oxygen it was thought that it might be a matter of some difficulty to prepare the substance in a sufficiently pure state for combustion.

It was therefore decided to prepare the reduction product of Z and to acetylate it

in one operation as in the method of FICHTER and WILLMANN (1904) for dialkylated dihydroxyquinones.

0.5 gm. Z, 2 c.c. acetic acid, and 1 gm. zinc dust, were therefore heated to boiling for about 1 minute. Then 10 c.c. of acetic anhydride were added and 4 gm. of sodium acetate. As the reduction was not quite complete, more zinc dust was added until the solution was practically colourless, this requiring 3 to 4 minutes heating. The product was poured into water, well stirred and filtered. A crystalline acetyl derivative was obtained mixed with zinc dust. The mixture was extracted with boiling absolute alcohol and filtered hot. There was a good separation of crystals on cooling. Yield = 0.7 gm. of an almost colourless product. When recrystallised from absolute alcohol it formed colourless rhombic prisms melting at 192.5° – 194° C.

This compound gave the following results on combustion :—

| Weight of Acetyl Compound Analysed. | Weight of H_2O . | Weight of CO_2 . | Percentage Hydrogen. | Percentage Carbon. |
|--|--------------------|--------------------|----------------------|--------------------|
| 0.1506 gm. | Gm. 0.0664 | Gm. 0.3011 | 4.93 | 54.52 |
| 0.1250 gm. | 0.0549 | 0.2476 | 4.91 | 54.03 |
| Theoretical for $C_8H_6O(O.CO.CH_3)_4$ | — | — | 5.10 | 54.23 |

The number of acetyl groups present was estimated as follows :—

0.2695 gm. of the acetyl compound was hydrolysed by boiling with 25 c.c. of N/1 H_2SO_4 in the same manner as was described above for diacetyl Z. The separation of the acetic acid formed on hydrolysis was also carried out in the same way.

30.00 c.c. of N/10 sodium hydroxide were required to neutralise the acetic acid formed, corresponding to 47.87 per cent. of $CH_3.CO$ in the acetyl compound. The calculated value for four acetyl groups in $C_8H_6O(O.CO.CH_3)_4$ is 48.59 per cent.

The reduced Z evidently contains four acetyltable hydroxyl groups. Since Z ($C_8H_8O_5$) itself contains two OH groups and gives rise to another two OH groups on reduction, the nature of four out of the five oxygen atoms in the molecule of Z has been determined. The nature of the only remaining oxygen atom was elucidated by carrying out an estimation of the alkoxyl groups present in Z.

Determination of alkoxyl groups in Z.—A ZEISEL estimation was carried out on Z with the following result :—

0.1948 gm. Z gave 0.2497 gm. silver iodide.

This expressed as methoxyl group = 16.92 per cent.

Calculated for 1 methoxyl group in $C_8H_8O_5$ = 16.85 per cent.

In order to decide whether the alkoxyl grouping is really a methoxyl group and not an ethoxyl or higher group, the products from a second ZEISEL operation were passed

into freshly distilled dimethylaniline. Crystals separated which were left overnight, and were then filtered off. They appeared to be plates, and on heating, sintered (in a sealed tube) at 210°C . and at about 220°C . they had completely volatilised. With quick heating they showed signs of melting at 230°C .

A little trimethylphenylammonium iodide was prepared from methyl iodide and dimethyl-aniline. It showed the same phenomena as the product from Z when an attempt was made to determine its melting point.

An iodine estimation was then carried out on the product from Z by precipitating the iodine with silver nitrate in aqueous solution in presence of HNO_3 . The silver iodide was collected on a Gooch crucible and weighed.

0.0833 gm. of the trialkylphenylammonium iodide from Z gave 0.0739 gm. of AgI , corresponding to 47.9 per cent. iodine.

Calc. for methyl compound, 48.3 per cent.

Calc. for ethyl compound, 45.8 per cent.

Calc. for propyl compound, 43.6 per cent.

The result agrees most closely with the methyl compound. Further support was obtained for the belief that the compound is really a methyl compound from the fact that a mixture of ethyl iodide and dimethylaniline could not be induced to form crystals. Methyl iodide gives crystals immediately as did the product from Z.

Z therefore contains one methoxyl group.

Action of hydroxylamine on Z.—0.2 gm. of Z, 0.5 gm. of hydroxylamine hydrochloride, and 15 c.c. of absolute alcohol were boiled under a reflux condenser for $1\frac{1}{2}$ hours. There was no separation of an oxime and 0.12 gm. of Z was recovered unchanged. It may be concluded, therefore, that Z does not form an oxime under these conditions.

Constitution of Z.—The evidence presented as to the constitution of Z may be summarized as follows :—

- (1) Z has the empirical formula $\text{C}_8\text{H}_8\text{O}_5$ and a molecular weight of 184 corresponding to this formula.
- (2) Z titrates as a dibasic acid.
- (3) Z contains in its molecule 5 oxygen atoms.

Of these :—

- (a) One oxygen atom is present as a methoxyl group.
- (b) Two other oxygen atoms are present as acetyltable hydroxyl groups, and, as Z titrates as a dibasic acid, these two OH groups are presumably phenolic OH groups attached directly to a benzene nucleus.
- (c) The remaining two oxygen atoms are present as CO groups since, on reduction,

they become acetyltable hydroxyl groups, reduced Z giving a tetra-acetyl compound.

(4) Z is purplish-black in colour, but becomes colourless on reduction. Z and its reduction product give an intense red-brown and blue-green colour with ferric chloride respectively.

(5) Z does not give an oxime with hydroxylamine.

(6) Z has a definite aromatic odour reminiscent of the quinones.

From all the above considerations we are of the opinion that Z is a hydroxyquinone derivative, with which class of bodies it agrees in all particulars. Further, since it titrates as a dibasic acid, both hydroxyl groups must be in the nucleus and not in a side chain, and Z is thus a dihydroxyquinone. This accounts for four carbon atoms of the six present in the benzene ring, *i.e.*, two as CO groups and two as C.(OH) groups. The other two carbon atoms must then be represented as either (a) CH and C.CH₂OCH₃ or (b) C.CH₃ and C.OCH₃.

The evidence available points to (a) as being very improbable for the following reasons:—

(1) It is known that hydroxyquinones containing an unsubstituted CH group in the nucleus behave in an abnormal manner when acetylated by acetic anhydride in the presence of concentrated sulphuric acid. Under these conditions the CH group as well as the CO groups and OH groups are acetylated to C.O.CO.CH₃. Thus a hydroxyquinone having a free CH group gives a tetra-acetyl derivative (THIELE and WINTER, 1900) whereas Z, which contains two OH groups gives only a diacetyl derivative under these conditions (see p. 250).

(2) It is known that hydroxyquinones are only "oximated" by hydroxylamine if they contain a CH group. Thus HOUBEN-WEYL, "Die Methoden der organischen Chemie" (2nd edition, Vol. 3, p. 611), say: "According to KEHRMANN the CO of halogen- and alkyl-substituted quinones can only be 'oximated' by hydroxylamine if there is at least one CH group in the *ortho* position to the CO group. The hydroxylated quinones conform to this rule in so far as hydroxylamine in hydrochloric acid solution is used. With alkaline hydroxylamine they show deviations." Since it has been shown that Z is not "oximated" by treatment with hydroxylamine hydrochloride the absence of a CH group in the nucleus is indicated.

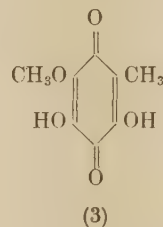
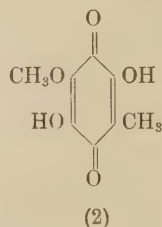
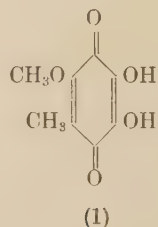
The six carbon atoms in the ring of the molecule of Z are thus arranged as follows:—Two carbon atoms as CO groups, two carbon atoms as C.(OH) groups, one carbon atom as a C.CH₃ group, and one carbon atom as a C.O.CH₃ group.

Z is thus a methoxy-dihydroxy-toluquinone.

There is at present no positive proof as to whether Z is a *para*- or *ortho*-quinone derivative, though the available evidence points to it being a *para*-derivative. The relative positions of the methyl, methoxyl, and two hydroxyl groups in the quinonoid

nucleus is also unknown since no compounds of this type have been described in the literature.

If it is allowed that Z is a *para*-quinone derivative it must have one of the following formulæ :—



Work is at present in progress having as its aim the synthesis of each of these compounds.

Note by Dr. CHARLES THOM, mycologist to the United States Department of Agriculture, on cultures Ad. 74, Ad. 79 and Ad. 80 :—

“ Our transfers of Ad. 74, Ad. 79 and Ad. 80 put them together and in the group with *P. spinulosum*, or, in deference to the belief that Dr. WEHMER's *Citromyces Pfefferianus* belonged somewhere in this group, to the *P. Pfefferianum* series. Our notes indicate that they approach *P. viridi-dorsum* of BIOÜRGE which, in our cultures from the transfer he furnished us, belongs here.”

Summary.

The products arising from the growth on a glucose medium of three strains of a species of *Penicillium* of the *P. spinulosum* series—Ad. 74, Ad. 79 and Ad. 80—have been investigated. All three strains produce large amounts of citric acid from glucose, but in addition give rise to smaller amounts of a substance “Z” which is responsible for the colour changes in the medium which are characteristic of this species of *Penicillium*. The preparation, properties and constitution of substance “Z” are described. Substance “Z,” which was separated as purplish-black permanganate-like crystals of the formula $C_8H_8O_5$, is a methoxy-dihydroxy-toluquinone. spin

This is the first recorded instance of the production from glucose of a quinone derivative by fungi.

Studies in the Biochemistry of Micro-organisms.

PART XIII.—*On a new type of mucilaginous material, Luteic acid, produced from Glucose by Penicillium luteum ZUKAL.*

By HAROLD RAISTRICK and MARGARET LESLEY RINTOUL.

The *Penicillium luteum*—*P. purpurogenum* group of species of *Penicillium* described by THOM (1915), contains a number of species and strains, with a strain of *P. luteum* ZUKAL, at the one end of the series and *P. purpurogenum* STOLL, at the other end.

The strain of *P. luteum* ZUKAL, which occupies one end of this series, “produces ascospores freely in all the media used and conidia very sparingly. In the actively growing culture the dominant shades of colour are yellow with tardy appearance of red.” *P. purpurogenum* STOLL, at the other end of the series, “produces only conidia, in which yellow shows transiently while red colours in mycelium and substratum are abundant.” “The production of yellow in the surface growth at some period of colony development or under some cultural conditions is typical for the group. This may be dominant, transient, or almost lacking, yet it is not difficult to demonstrate in the organisms studied. Coincident with the change of colour in the surface or aerial growth we find at the *luteum* end of the series that yellow to orange shades predominate in the substratum. These slowly or but partially change to red as the colonies become old. In the forms producing conidia only, yellow or orange tones still appear in the young colony. The change to red is slow and only partial in some forms, but towards the *purpurogenum* end of the series the yellow colours are reduced to but transient appearances, replaced quickly and almost completely by red.” (The quotations are from THOM’s paper quoted above.)

The biochemical characteristics of the different species also vary considerably. Thus the acidity produced when the different species are grown on glucose solutions varies from negligible amounts at the *luteum* end of the series to extraordinarily high yields of acid—gluconic acid—by species at the *purpurogenum* end of the series. The strain *P. purpurogenum* var. *rubrisclerotium* THOM, No. 2670, mentioned by MAY, HERRICK, THOM and CHURCH (1927), gives yields of gluconic acid approximating 80 per cent. of the theoretical. The strains of *P. luteum*, giving low yields of acid, also give rise to extremely mucilaginous solutions, and the experiments described in this paper deal with the isolation and investigation of this mucilage.

The history of the culture of *P. luteum* ZUKAL, used in this work is as follows :—

P. luteum ZUKAL, Catalogue No. Ad. 30, obtained from the Centraalbureau voor Schimmelcultures at Baarn on 28th May, 1925.

Dr. CHARLES THOM, to whom this culture was sent for examination, wrote as follows: "No. Ad. 30 is apparently a member of the *P. luteum* group. In this case we also run up against a peculiar history, as you know. The original culture which I reported as *P. luteum*, No. 11, has been maintained for 20 years as an ascosporic form received from Prof. THAXTER, carried to Prof. WEHMER, labelled and agreed to by him as this species. The validity of this species is attacked by DERX and BIOURGE. Several times in the period during which I have kept it in culture it has separated into two lines, one of which is pale in colour, very little yellow either above or below and produces no ascospores. This form harmonises with Ad. 30 as I have received it. This strain would, in a way, satisfy DERX' contention that ascosporic forms in this series are produced by the conjugation of two strains which are separately non-ascosporic."

The following are the salient points of the carbon balance sheet prepared in metabolism experiment F 12 on Ad. 30 (see Part IV):—

- (1) Residual sugar: (a) By polarimeter, 0.275 per cent.; (b) by SHAFFER and HARTMANN, 0.387 per cent.; (c) by alkaline iodine, 0.454 per cent.
- (2) Increase in acidity, 0.6 c.c. N/1 acid per 250 c.c. medium.
- (3) Carbon precipitated as calcium salt by 80 per cent. alcohol, 0.477 gm.
- (4) Carbon as CO₂ in solution, volatile neutral compounds, volatile acids and "carbon unaccounted for," were all negligible.
- (5) Carbon as synthetic carbon in colloidal iron precipitate, 0.265 gm.

The indications from this balance sheet are that Ad. 30 forms a lævorotatory substance which is precipitated from strong alcohol as a calcium salt and which is precipitated, at any rate in part, by colloidal iron.

As a preliminary step in the isolation of the mucilaginous substance from cultures of Ad. 30 a number of test tube cultures, plugged with cotton wool, of this mould were made in 10 c.c. quantities of the usual CZAPEK-DOX solution containing 5 per cent. of glucose. These were incubated at 24° C. and tested periodically. The following general observations were made. The culture grew well at this temperature, giving rise to a white mycelium which showed very little sign of sporing. The metabolism solution became pale yellow in colour and very sticky in nature. It filtered quite clear, however, although very slowly. The sticky filtrate gave the following qualitative tests:—

- (1) No coagulation on heating.
- (2) Saturation with ammonium sulphate gave a sticky precipitate which coalesced and adhered to the sides of the tube.
- (3) Addition of a large volume of alcohol gave rise to a white cloud which on shaking produced a white flocculent precipitate.

- (4) Ferric chloride or basic lead acetate gave rise to heavy gelatinous precipitates.
- (5) Biuret test negative.

These tests indicated that the substance was probably not protein in nature. Since the filtrate from the alcohol precipitate in test 3 and from the ferric chloride and basic lead precipitates in test 4 had lost all sign of stickiness, these precipitants seemed to offer methods of isolation. Comparison of alcohol and basic lead as precipitants indicated that alcohol was the more suitable. The work subsequently carried out will now be described under the following four main headings :—

- (1) Preparation and general characteristics of crude material.
 - (2) Purification of crude material and properties of pure product, luteic acid.
 - (3) Acid hydrolysis of luteic acid and examination of hydrolytic products.
 - (4) Alkaline hydrolysis of luteic acid and examination of hydrolytic products.
- Preparation of luteose.

SECTION 1.—*Preparation and general characteristics of crude material.*

The large scale incubator described on p. 136 (Part VII), containing 60 litres of CZAPEK-DOX 5 per cent. glucose distributed in 12 trays, was sown on 29th July, 1927, with a spore suspension from 6 ROUX bottle cultures on CZAPEK-DOX agar, 5 ROUX bottle cultures on beer wort agar and 20 CZAPEK-DOX agar slopes in test tubes. The beer wort cultures provided very poor sporing growth, the suspension from these bottles containing mostly mycelium. Better growth and sporing was obtained in the CZAPEK-DOX agar cultures, although these were not very good. After mixing as usual with an aluminium stirrer the incubator was kept at room temperature. Aeration was started on 2nd August, 1927, and continued through the whole course of incubation. A sample from tray 8 was taken on 12th August, 1927 (after 14 days' incubation). The filtered solution contained 1.43 per cent. glucose by polarimeter and gave typical reactions with ferric chloride, basic lead acetate, alcohol and ammonium sulphate. Further samples were taken on 18th August, 1927, from trays 4 and 8.

They each gave quite good qualitative tests and the following figures for glucose :—

| Sample. | | | | | Percentage Glucose by Polarimeter. | Percentage Glucose by Alkaline Iodine. | Difference. |
|---------|-----|-----|-----|-----|--|---|-------------|
| Tray 4 | ... | ... | ... | ... | 0.165 | 0.615 | 0.450 |
| Tray 8 | ... | ... | ... | ... | 0.098 | 0.547 | 0.449 |

Trays 7 to 12 were taken off on 18th August and trays 1 to 6 on the following day. As much liquid as possible was drained from the mycelium and filtered. The mycelium

itself, which was very thick and white and intensely slimy to the touch, was squeezed dry through muslin, filtered and the filtrate combined with the main filtrate. The combined filtrates were then evaporated in trays in a current of warm air to about 5 litres and an equal volume of alcohol added. By this means a large quantity of very sticky, toffee-like, precipitate was obtained. This was dissolved in water giving a very mucilaginous solution which was very difficult to filter. It was finally dealt with by diluting somewhat and filtering through a number of kieselguhr filters, giving a practically clear yellowish brown filtrate. To this was added a volume and a half of alcohol, giving rise to a white suspension which did not settle overnight. Tests on small quantities of this suspension showed that it was difficult to break up with alcohol alone, but that the addition of ether threw down the substance fairly completely. Hence one volume of ether was added with constant stirring to the alcohol-water suspension. The material separated as a pale yellowish brown mass similar to a soft toffee in appearance. This was spread in thin layers in dishes and dried in a vacuum desiccator. After drying the crude material was powdered and thus appeared as a pale yellowish brown amorphous solid. The yield of crude material was about 350 gm. (about 12 per cent.).

In a second preparation of this material, carried out later, 280 gm. of pure product were obtained (about 9 per cent.).

As no methods of purification were at the time available, and as all attempts to crystallise the material failed, the experiments about to be described were carried out in order to obtain some information as to its general properties. (Note that all these experiments were carried out on the crude material prepared as just described.)

(1) *Ash determination.*—2.5016 gm. of the material dried *in vacuo* were ashed to constant weight. Weight of ash 0.2787 gm. equal to 11.14 per cent.

Qualitative tests showed that the ash consisted principally of magnesium carbonate and magnesium phosphate.

(2) *Matter volatile at 100° C.*—2.4131 gm. were dried to constant weight at 100° C. in air. The loss in weight was 0.2875 gm. equal to 11.92 per cent. This was apparently mostly alcohol.

(3) *Optical rotation of crude material.*—The crude material was strongly lævorotatory and gave values for $[\alpha]_{\text{Hg, green}}$ in 2 per cent. aqueous solution of -24.9° , -26.7° , -27.7° , -29.9° . These values were obtained at different times and the lack of agreement between them is probably due to the varying amount of volatile matter present.

(4) *Effect of enzymes.*—200 c.c. of a 1.996 per cent. solution of the crude material having a rotation of -1.193° in a 20 cm. tube, corresponding to $[\alpha]_{\text{Hg, green}}$ of -29.9° , were used for testing the effect of invertase and diastase. 50 c.c. of this solution were treated with 10 c.c. of commercial invertase solution (Difco brand). Another 50 c.c. of solution were treated with 0.1 gm. of commercial diastase (Pangestin Difco brand). A little toluene was added to each flask and these, together with blanks,

were incubated at 37° C. The effect of the enzymes was followed polarimetrically with the following results :—

| Enzyme used. | Incubation Period. | °Rotation in 20 cm. Tube. | Initial °Rotation. |
|------------------|------------------------|---------------------------|--------------------|
| Invertase | 3 $\frac{3}{4}$ hours | — 0·927 | } — 0·994 |
| „ | 22 hours | — 0·910 | |
| „ | 3 days | — 0·926 | |
| Diastase | 4 $\frac{3}{4}$ hours | — 1·202 | } — 1·193 |
| „ | 22 $\frac{1}{2}$ hours | — 1·186 | |
| „ | 3 days | — 1·263 | |

It is evident from these results that the material was not hydrolysed by either diastase or invertase.

(5) *Hydrolysis with acid.*—The crude material gave very little, if any, reduction of BENEDICT'S solution. After boiling with N/1 H₂SO₄ for some time the resultant solution gave a copious reduction of BENEDICT'S solution. Hydrolysis had obviously taken place and the progress of hydrolysis was followed quantitatively as follows :—10 gm. of crude material were dissolved in the cold in 500 c.c. of N/1 H₂SO₄. The solution was immediately polarised in a 20 cm. tube and then heated on a boiling water bath, and samples taken at intervals and polarised with the following results :—

| Time of Hydrolysis. | °Rotation in 20 cm. Tube. |
|----------------------|---------------------------|
| Zero | — 0·997 |
| 15 mins. | — 0·380 |
| 30 mins. | — 0·187 |
| 1 hr. | + 0·143 |
| 1 $\frac{1}{2}$ hrs. | + 0·480 |
| 2 hrs. 10 mins. | + 0·956 |
| 3 hrs. 15 mins. | + 1·297 |
| 4 hrs. 15 mins. | + 1·475 |
| 5 hrs. 15 mins. | + 1·568 |
| 7 hrs. | + 1·590 |

(6) *Products of acid hydrolysis.*—A little of the above hydrolysis solution on heating with phenylhydrazine acetate and sodium acetate for about 1 hour gave a yellow osazone in small needle-shaped crystals similar in appearance to glucosazone. In order to identify this definitely as glucosazone 10 gm. of the crude material were hydrolysed with 500 c.c. of N/1 sulphuric acid on a boiling water-bath for five hours and neutralised with N/1 barium hydroxide. After filtration the solution was heated on a water-bath with 10 gm. of phenylhydrazine, 5·5 gm. of acetic acid and 10 gm. of sodium acetate.

The osazone was collected in four crops, giving a total yield of 12 gm. It was purified by precipitation from pyridine (see NEUBERG, 1899). The optical rotation of various fractions of the osazone in pyridine-alcohol solution according to NEUBERG's method was then determined. 0.2 gm. of dry osazone was dissolved in 4 c.c. of pure pyridine, 6 c.c. of absolute alcohol added and the solution polarised in a 10 cm. tube with the following results :—

| Sample of Osazone. | °Rotation in 10 cm. Tube. Mercury Yellow Line. |
|--|--|
| 1st crop | — 1.474 |
| 2nd crop | — 1.365 |
| 3rd crop | — 1.334 |
| 4th crop | — 1.485 |
| Sample of pure glucosazone prepared from pure glucose ... | — 1.475 |

These results indicate that the only sugar formed by the hydrolysis of this material was probably glucose. Consideration of the yields of glucose obtained seemed to indicate that glucose was not the only product of hydrolysis and with a view to settling this point experiments were now undertaken to obtain as pure a sample of the material as possible. These are described under Section 2.

SECTION 2.—*Purification of crude material and properties of luteic acid.*

The analytical figures given on p. 258 show that the crude material contained an abnormal amount of ash. An attempt was made to reduce this to a minimum. It was hoped that, because of the mucilaginous nature of the crude material, it might be possible to dialyse away the mineral matter present. With this end in view collodion dialysing tubes were made in large boiling tubes. A 5 per cent. solution of the crude material was made and 150 c.c. quantities placed in the dialysing tubes, the open ends of which were closed by corks and made watertight with a layer of collodion. They were then dialysed against running tap water. Samples were taken out after two days' and four days' dialysis respectively and ash determinations carried out. The ash content was 8.36 per cent. in the first case and 7.20 per cent. in the second case. The dialysis was stopped after four days since microscopic examination showed that the solutions had become slightly infected. The material was recovered by evaporation and precipitation with alcohol. A quantity of this material was dried and ashed and a quantitative examination of the ash made. The ash amounted to 7.64 per cent. of the dry material, was practically free from phosphate, and consisted almost entirely of magnesium carbonate or oxide. It thus appeared probable that the *crude material*

was in reality a magnesium salt of an organic acid. Hence about 30 gm. of the crude material were dissolved in 500 c.c. of water acidified with 15 c.c. of concentrated hydrochloric acid. Complete solution was thus obtained and alcohol was now added to complete precipitation. The precipitate was considerably lighter in colour than that previously obtained and was not quite so sticky. It was redissolved in water and reprecipitated with alcohol, and a portion of this was dried in a vacuum desiccator and ashed. The percentage of ash was now only 1.14 per cent. The main bulk (23 gm.) was redissolved in 250 c.c. of water and acidified with 1 c.c. of concentrated hydrochloric acid and reprecipitated with alcohol. This time the material did not coagulate and form a uniform mass nearly so readily as it had done previously. It was again redissolved in 250 c.c. of water, the solution filtered and alcohol added. The addition of two volumes of alcohol, however, though giving rise to a very dense white suspension, did not produce an appreciable separation. Two volumes of ether were added, with constant stirring, and on allowing to stand a pure white precipitate settled out. This was filtered as quickly as possible on a Buchner funnel and washed with ether without ever being allowed to dry. It was finally drained and placed in a desiccator which was immediately evacuated. It dried quickly to a white mass, rather like starch, which could be quite easily ground to a fine white amorphous powder. This material constitutes the pure product on which all subsequent experiments were carried out, and to which we propose to give the name "luteic acid."

Properties of luteic acid.—Luteic acid is a fine white, somewhat hygroscopic and amorphous powder. Its aqueous solution is strongly acid to litmus, so that it seems evident that the crude material was in reality a magnesium salt of an acid which is now designated luteic acid. Luteic acid holds tenaciously small amounts of the solvents used in its purification, and for the subsequent analytical work all samples were dried to constant weight at 100° C. in a current of dry nitrogen. By this means no alteration in colour was produced, whereas drying at 100° C. in air gives rise to a yellowish product.

Ash determination.—0.5061 gm. gave 0.0040 gm. of ash equal to 0.79 per cent.

Equivalent of luteic acid.—0.5699 gm. of luteic acid was dissolved in water and titrated with N/10 sodium hydroxide to phenolphthalein. 13.11 c.c. of N/10 sodium hydroxide were needed for neutralisation. This corresponds to an equivalent of 434.7.

Optical rotation.—An attempt was made to determine the optical rotation of luteic acid itself, but on account of the gelatinous nature of the solution this had to be abandoned and only an approximate value of the optical rotation of the sodium salt was obtained. 0.9942 gm. of the dried material was weighed out into a 500 c.c. measuring flask. The addition of about 100 c.c. of water produced a stiff gel. Addition of more water gave a very viscous liquid which did not seem to be a true solution as it was not homogeneous even after standing overnight. The calculated amount of N/10 sodium hydroxide for neutralisation was then added and the volume made up to 500 c.c. By this means a true solution was formed, of approximately 0.2 per cent. concentration, of

the sodium salt, but even this was still so viscous that centrifuging was necessary to remove bubbles before it could be polarised. The optical rotation in a 20 cm. tube with mercury green light was -0.187° corresponding to $[\alpha]_{\text{Hg. green}} = -47^\circ$.

Action of Alkaline Iodine.—0.0718 gm. of luteic acid, dried to constant weight, was treated with alkaline iodine solution. After standing for two hours only 0.47 c.c. of N/10 iodine had been absorbed. It can therefore be concluded that the substance does not react with alkaline iodine and hence contains no free CHO groups. This conclusion is supported by the fact that the substance did not reduce BENEDICT'S solution, nor did it form an osazone with phenylhydrazine.

SECTION 3.—*Acid hydrolysis of luteic acid and examination of hydrolytic products.*

10 gm. of luteic acid were hydrolysed with 500 c.c. of N/1 sulphuric acid on a boiling water-bath for about nine hours. The sulphuric acid was removed quantitatively with pure barium hydroxide. The filtrate from the barium sulphate precipitate measured approximately one litre and was strongly acid in reaction. 10 c.c. of this solution required 4.49 c.c. of N/10 sodium hydroxide for neutralisation corresponding to approximately 45 c.c. of N/1 sodium hydroxide for the whole solution. Since 10 gm. of material would require only about 23 c.c. of N/1 sodium hydroxide for neutralisation before hydrolysis, it is evident that the acidity had been doubled during hydrolysis. The acid filtrate was now shaken with excess of pure precipitated calcium carbonate at a temperature of 50° — 60° C. until the solution was permanently neutral in reaction. It was then filtered and the clear filtrate evaporated *in vacuo* at low temperature to about 50 c.c. During evaporation a quantity of white needle-shaped crystals separated (Fraction A). The filtrate from these was evaporated to 10 c.c. and 100 c.c. of pure methyl alcohol were added in portions, giving rise to a brownish flocculent precipitate (Fraction B). This was centrifuged off, washed with methyl alcohol and the methyl alcohol solution evaporated *in vacuo* to a sticky syrup which, on standing, set to a mass of yellowish brown crystals (Fraction C).

Treatment of Fraction A.

Weight = 1.5 gm.—Fraction A was recrystallised by dissolving in excess of water at about 50° C. and evaporating the solution *in vacuo*. This gave rise to a mass of white needles which were filtered off, washed and air dried. Filtrate and washings were combined with Fraction B. The calcium salt was only slightly soluble in cold water, moderately so in warm and appeared to separate in a different crystalline form from boiling water. It did not give any colour reaction with ferric chloride. A portion of the calcium salt which had been standing *in vacuo* over strong sulphuric acid was dried to constant weight at 110° C. It lost 6.99 per cent. on so drying.

0.1870 gm. of the material dried at 110° C. was heated to constant weight over a blow pipe. The weight of the residual calcium oxide, which was quite white in colour,

was 0.0707 gm., corresponding to 27.03 per cent. of calcium in the original calcium salt and a molecular weight of the free acid of 55 if monobasic, 110 if dibasic and 165 if tribasic.

Treatment of Fraction B.

Weight = 0.56 gm.—Fraction B was dissolved in about 100 c.c. of warm water giving a clear brownish solution. This solution was decolorised with a little blood charcoal, combined with the filtrate from Fraction A, and evaporated *in vacuo*. A further quantity of needle crystals was obtained. These were shown to be identical with the calcium salt in Fraction A and no other product was obtained from the mother-liquors.

Treatment of Fraction C.

Weight = 8.48 gm.—This material was divided into two parts and treated as follows :—

(a) About 3 gm. were recrystallised from absolute methyl alcohol from which it separated in pure white prismatic needles having the following characteristics :—(1) Melting point 149°C .; (2) 0.7573 gm. dried to constant weight at 110°C . was dissolved in 99.7 c.c. of water. Part of this, polarised as quickly as possible, gave a rotation of $+3.56^{\circ}$ in a 40 cm. tube. Another portion to which one drop of N/1 sodium hydroxide was added gave a *steady* rotation of $+1.90^{\circ}$ in a 40 cm. tube. These correspond to an initial $[\alpha]_{\text{Hg. green}} = +117.2^{\circ}$ and a final $[\alpha]_{\text{Hg. green}} = +62.5^{\circ}$; (3) an alkaline iodine estimation gave an estimated glucose content of 0.7479 per cent. on solution of (2).

(b) A portion was treated with phenylhydrazine and gave rise to an osazone which separated from the boiling solution in yellow needles having a melting point of 199°C . and a rotation in pyridine and absolute alcohol solution of -1.52° in a 10 cm. tube, using the mercury green line. The observed value for pure glucosazone is -1.51° .

All the above figures leave no doubt that Fraction C consisted entirely of glucose. The only products of hydrolysis were thus glucose and an acid, the calcium salt of which constituted Fractions A and B. This acid was identified as malonic acid by the following tests : 1.114 gm. of the air-dried calcium salt were treated with 0.8835 gm. of hydrated oxalic acid. The calcium salt was suspended in warm water and shaken with the oxalic acid for about an hour. The calcium oxalate was filtered off and the aqueous solution evaporated *in vacuo*. There was no separation until the volume had been reduced to about 1 to 2 c.c., when crystalline crusts began to separate round the edge of the liquid. The solution was dried down in a vacuum desiccator and the crude acid sublimed in a high vacuum. The acid readily sublimed at $110^{\circ} - 120^{\circ}\text{C}$. forming a white sublimate consisting of prisms mostly combined in rosettes. It had the following properties :—

(a) It was very soluble in water, giving an intensely acid solution which did not reduce potassium permanganate in the cold, gave no colour reaction with ferric

chloride and no precipitate with 2:4-dinitrophenylhydrazine or with calcium acetate solution.

(b) It melted at 135° — 135.5° C. after softening at 133° C. Decomposition with gas evolution took place at the melting point. Admixture with a sublimed sample of synthetic malonic acid produced no lowering of the melting point.

(c) A few milligrammes heated with 1 c.c. of acetic anhydride gave a greenish yellow colour, showing also a greenish fluorescence which became more marked on the addition of 1 c.c. of glacial acetic acid. This test is held to be specific for malonic acid.

(d) 0.0349 gm. of sublimed material required 6.77 c.c. N/10 sodium hydroxide for neutralisation to phenolphthalein, corresponding to a combining weight of 51.6. Malonic acid has a combining weight of 52.

(e) The sample gave the following figures on combustion:—

| Weight of Substance. | Weight of CO ₂ . | Weight of Water. | Percentage Carbon. | Percentage Hydrogen. |
|--|-----------------------------|------------------|--------------------|----------------------|
| 0.1532 gm. | 0.1946 | 0.0559 | 34.63 | 4.08 |
| 0.1204 gm. | 0.1516 | 0.0240 | 34.35 | 3.90 |
| Theoretical for malonic acid C ₃ H ₄ O ₄ | — | — | 34.60 | 3.88 |

Having shown that the only hydrolytic products were glucose and malonic acid it seemed desirable to obtain some information as to the relative proportions of these two compounds in the original molecule. The first step towards this was to estimate the amount of glucose produced on hydrolysis, and this was carried out as follows:— 1.0233 gm. of luteic acid dried in nitrogen to constant weight and corresponding to 1.0132 gm. of ash-free material were hydrolysed by heating on a water-bath with 50 c.c. of N/1 sulphuric acid for $8\frac{1}{2}$ hours. At the end of that time the solution was made up accurately to 99.76 c.c. The glucose content of this solution was then estimated by:—

(a) *Polarimeter*.—Percentage of glucose in solution was 0.8395, corresponding to 82.7 gm. of glucose from 100 gm. of the original ash-free material.

(b) *Wood-Ost method*.—Percentage of glucose in solution was 0.846, corresponding to 83.3 gm. of glucose from 100 gm. of the original ash-free material.

The amount of malonic acid produced on hydrolysis has not been specifically determined, but may be deduced approximately from the following consideration. On p. 262 it is noted that the hydrolytic products from 10 gm. of purified material required 45 c.c. of N/1 sodium hydroxide for neutralisation. This corresponds to a yield of 23.4 gm. of malonic acid from 100 gm. of material.

SECTION 4.—*Alkaline hydrolysis of luteic acid and examination of hydrolytic products.**Preparation of luteose.*

10 gm. of luteic acid were hydrolysed by boiling for $1\frac{1}{2}$ hours under reflux with 300 c.c. of N/4 barium hydroxide. During the hydrolysis there was a copious separation of barium malonate which, at the end of the hydrolysis, was filtered off. The hydrolysis mixture was made up to 500 c.c., 25 c.c. of concentrated HCl added with constant shaking, and then 550 c.c. of 96 per cent. alcohol. There was a copious separation of a pure white amorphous material. The mixture was left at 0° C. and the precipitate filtered off later. It was then redissolved in 150–200 c.c. of hot water, cooled, 2 c.c. of concentrated HCl added, and then an equal volume of alcohol. The resultant precipitate was filtered, washed and again dissolved in hot water from which it was precipitated by the addition of an equal volume of alcohol, but in this case no acid was added. The precipitated material, to which we propose to give the name “luteose,” was filtered off, washed with alcohol and ether and dried in a desiccator.

Luteose was shown to be a complex polysaccharide and had the following properties: It consisted of a pure white, light amorphous powder, which is appreciably soluble in cold water, and readily soluble in hot water giving a clear colourless solution which on cooling becomes opalescent, and in moderately strong solutions is very viscous. The aqueous solution gives no colour with iodine.

The following estimations were carried out on samples of luteose dried to constant weight *in vacuo*.

1. *Ash*.—0.1815 gm. gave 0.0003 gm. ash corresponding to 0.16 per cent.

2. *Acidity*.—Luteose is definitely neutral in reaction, since with 0.2351 gm. a single drop of N/10 sodium hydroxide was sufficient to render the solution alkaline to phenolphthalein.

3. *Optical Rotation*.—Luteose is markedly laevorotatory. Because of the fact that its aqueous solution is slightly opalescent in the cold the optical rotation was determined in a jacketed tube at 90° C. 0.5248 gm. was dissolved in 50 c.c. of water and polarised in a 20 cm. tube at 90° C. The average of a large number of readings obtained was -0.886° for the mercury yellow light corresponding to $[\alpha]_{\text{Hg, yellow}}^{90^{\circ}\text{C.}} = -42.2^{\circ}$, and -0.974° for the mercury green light corresponding to $[\alpha]_{\text{Hg, green}}^{90^{\circ}\text{C.}} = -46.4^{\circ}$.

4. *Hydrolysis by boiling dilute acid*.—0.4819 gm. of luteose was hydrolysed by boiling for six hours with 10.03 c.c. of N/1 H_2SO_4 . The hydrolysis mixture was cooled and titrated with N/1 NaOH of which 10.10 c.c. were required, thus indicating that no acid was formed during the hydrolysis. The mixture was made up to 49.92 c.c., filtered, and the glucose content estimated:—

(a) By polarimeter = 0.967 per cent.

(b) By Wood-Ost method = 0.964 per cent.

Since 0.4819 gm. dissolved in 49.92 c.c. corresponds to 0.965 per cent. only small amounts of any hydrolytic product other than glucose can have been formed. This

was finally proved by converting the hydrolysis product into the osazone by treatment with phenylhydrazine and polarising the solution of the osazone in pyridine and ethyl alcohol. 0.2 gm. of the osazone (M. Pt. 205° C.) dissolved in a mixture of 4 c.c. of pyridine and 10 c.c. of ethyl alcohol gave a rotation of -1.35° (Observed value for pure glucosazone = -1.50° .)

5. *Combustion results.*—The following results were obtained on combustion (SCHOELLER, Berlin), on a sample dried to constant weight *in vacuo* over P_2O_5 at 50° C. :—

| Weight of substance analysed. | Weight of carbon dioxide. | Weight of water. | Percentage C. | Percentage H. |
|------------------------------------|---------------------------|------------------|---------------|---------------|
| 3.993 mgm. (0.015 mgm. residue) | mgm. 6.345 | mgm. 2.31 | 43.51 | 6.50 |
| 4.374 mgm. (0.022 mgm. residue) | 6.950 | 2.54 | 43.56 | 6.53 |

We desire to express our thanks to Prof. T. M. LOWRY and Mr. MORTON for the observation that luteic acid on hydrolysis with dilute barium hydroxide gives rise to the acid-free polysaccharide, luteose, described above.

Discussion of results obtained.

The following results are of significance in attempting to obtain some idea of the constitution of luteic acid :—

- (a) It is an acid having a combining weight of 434.7 (p. 261).
- (b) It does not react with alkaline iodine, does not reduce BENEDICT'S solution, nor form an osazone with phenylhydrazine (p. 262). These observations show that the material contains no free CHO groups.
- (c) It is lævorotatory (p. 261).
- (d) It is not hydrolysed either by diastase or invertase.
- (e) It is hydrolysed by acids giving glucose and malonic acid as the only products of hydrolysis (pp. 262–264).
- (f) The original acidity of the material is doubled on acid hydrolysis (p. 262). This indicates that for every free COOH group present in the original material another COOH group is freed during hydrolysis.
- (g) On acid hydrolysis 100 gm. of luteic acid give rise to about 83 gm. of glucose and 23.4 gm. of malonic acid (p. 264). A compound of two molecules of glucose condensed with one molecule of malonic acid with the loss of two molecules of water would, by hydrolysis of 100 gm., give rise to 84.1 gm. of glucose and 24.3 gm. of malonic acid.
- (h) On alkaline hydrolysis with dilute barium hydroxide, luteic acid gives rise to malonic acid and a complex polysaccharide, luteose, which is neutral in reaction and which is lævorotatory (p. 265).

Bearing all the above results in mind it seems probable that luteic acid is a complex compound, each molecule of which is built up of a number of similar units. Each unit in its turn is a condensation product of two molecules of glucose with one molecule of malonic acid, with the loss of two molecules of water, in which one COOH group is free while the other is in combination, and in which the two CHO groups are linked in such a way as to destroy their aldehydic properties. Such a substance would have a combining weight of 428, its acidity would double on hydrolysis, and 100 gm. would give rise to 84.1 gm. of glucose and 24.3 gm. of malonic acid.

Since alkaline hydrolysis liberates all the malonic acid and produces a new polyglucose which has obviously a high molecular weight it appears that luteic acid is a complex malonyl polyglucose, perhaps somewhat similar in structure to acetyl cellulose or nitrocellulose.

Natural polysaccharides of this type, which give rise on hydrolysis to a simple acid in addition to glucose, are very rare. Curiously enough the only other well-authenticated example is also of micro-biological origin. The American workers HEIDELBERGER and GOEBEL (1927) have recently shown that in the metabolism solution of young cultures of *Pneumococcus* there is present a substance which they designated the "soluble specific substance," which has the remarkable property of being precipitated from solution by an anti-pneumococcal serum prepared from the same type of *Pneumococcus*. The same substance was also found in the blood of animals infected with *Pneumococcus*. This "soluble specific substance," which is very similar in physical properties to luteic acid gives on hydrolysis glucose and glucuronic acid. Thus the main chemical difference between these two substances is in the nature of the acids produced by hydrolysis.

The natural gums, *e.g.*, gum arabic, gum tragacanth, etc., are, of course, of a somewhat similar chemical nature, since the work of O'SULLIVAN has shown that they are complex acids built up by the condensation of pentoses and hexoses with an apparently complex acid, the nature of which has not been worked out.

Summary.

Penicillium luteum ZUKAL (non-ascospore strain) produces as the result of its growth on glucose a mucilaginous material, luteic acid, the nature and properties of which have been investigated. This substance, the salts of which give rise to very viscous solutions, is a colloidal material of high molecular weight built up of unusual polysaccharide units, each of which is a product arising from the condensation, by the loss of two molecules of water, of two molecules of glucose with one molecule of malonic acid in such a way as to leave only one carboxyl group of the latter free. On alkaline hydrolysis luteic acid gives rise to a neutral, lævorotatory poly-glucose, for which the name luteose is proposed.

Studies in the Biochemistry of Micro-organisms.

PART XIV.—*On the production and chemical constitution of a new yellow colouring matter, citrinin, produced from glucose by Penicillium citrinum THOM.*

By ARTHUR CLEMENT HETHERINGTON *and* HAROLD RAISTRICK.

It has been known for some time that *Penicillium citrinum* THOM when grown on suitable media produces a characteristic lemon yellow colour. Thus THOM in his monograph on "Cultural Studies of species of *Penicillium*" (1910), says, "*P. citrinum* produces a lemon yellow colour, soluble in alcohol, in media containing sugars, milk, gelatin, bread and potato." This colouring matter was not isolated, however, nor was its nature further investigated.

In the course of an investigation into the colour reactions given with ferric chloride by metabolism solutions produced by various species of *Penicillium*, it was found that a very characteristic reaction was given by each of the strains of *Penicillium citrinum* THOM in our possession, but was not given by any other species in pure culture whether of *Penicillium* or of any other genus tested.

The three strains of *P. citrinum* THOM used in this work were sent to Dr. CHARLES THOM for his opinion. Their history is as follows :—

1. *P. citrinum* THOM. Catalogue Number Ad. 23, received from Mr. F. T. BROOKS, of Cambridge, in 1925. Dr. THOM says of this culture, "Ad. 23 varies somewhat in structure from the usual forms of *P. citrinum* while harmonising in general reactions with that species. In microscopic examination and cultural reactions, however, it is closest to a strain received from Baarn and carried as 4876·24 but labelled by Dr. WESTERDIJK as *P. fellutanum* BOURGE. As this organism was placed in my original draft of the manuscript for my book, it would have been very near to *P. citrinum*, but because the more frequent type of conidial mass was monoverticillate, I transferred it in the final write-up to that section, cross-referencing it to indicate its possible relationship to the *P. citrinum* series."
2. *P. citrinum* THOM. Catalogue No. Ad. 95. This strain is Dr. THOM's own culture No. 4733·14, and was purchased from the American Type Culture

Collection, Catalogue No. 1109. Dr. THOM says of this culture, "No. 95 is the organism Miss CHURCH sent to you and to Mr. BROOKS as *P. citrinum*."

3. *P. citrinum* THOM. Catalogue No. Ad. 114. This strain was received from Mr. L. D. GALLOWAY, of the Shirley Institute of the Cotton Research Association, under the label *P. aurifluum* (No. 94), which, according to BIOURGE, is almost synonymous with *P. citrinum*. Dr. THOM says of this organism, "No. 114 is another of the variant forms but is near enough to this group to be left in it."

When each of these three strains is grown on a modified CZAPEK-DOX glucose medium containing twenty times the amount of ferrous sulphate given in Part I, p. 7, in vessels having an ample supply of air, *e.g.* in flasks loosely plugged with cotton wool, the metabolism solution gradually takes on a yellow colour which deepens in tone until it becomes orange yellow. The filtered metabolism solution which is orange yellow in colour gives the following reactions:—

(1) Addition of ferric chloride solution gives first a heavy buff coloured precipitate which dissolves in excess of the reagent to give a very intense iodine-brown coloured solution.

(2) Potassium permanganate solution is immediately decolorised giving rise to a solution which no longer gives a colour with ferric chloride.

(3) Acidification by means of HCl or H₂SO₄ causes immediate discharge of the orange yellow colour of the medium and the deposition on standing of a yellow micro-crystalline powder having a characteristic appearance under the microscope.

These crystals consist of yellow, flat, narrow, elliptical plates having pointed ends and serrated edges and being usually combined in groups of two or three by their extremities. This substance to which we propose to give the name "citrinin" appears to be characteristic of *P. citrinum* THOM, since, apart from the above three strains of *Penicillium citrinum*, no other species of *Penicillium* nor indeed of any other genus of fungi amongst the 400 to 500 species tested gives the typical ferric chloride reaction or the equally typical micro-crystalline deposit on acidification. Of the above three strains of *P. citrinum* the best yields of citrinin are given by the strain Catalogue No. Ad. 23, though considerable quantities have been prepared from each of the other two strains Catalogue Nos. Ad. 95 and Ad. 114.

The preparation in fair quantity of this substance and the investigation of its chemical properties and constitution are given in the following paper.

Preparation of citrinin.

Thirty litres of CZAPEK-DOX glucose solution were made and distributed in 350 c.c. quantities in 85 1-litre conical flasks plugged with cotton wool. After sterilizing by steaming for half an hour on each of three consecutive days, the flasks were sown with a suspension of spores of *P. citrinum* Ad. 23 in distilled water, prepared from Roux

bottle cultures on beer wort agar. The flasks were then incubated at 28° C. until the production of citrinin had reached a maximum.

A rough measure of the amount of citrinin present is given by the figure obtained on subtracting the percentage of glucose present as estimated by the polarimeter, from the percentage of glucose present as estimated by alkaline iodine. This figure, however, is, at the best, only an approximation to the true amount of citrinin present, since citrinin itself is lævorotatory. It will be shown on p. 276 that citrinin is readily acted on by alkaline iodine. The point of maximum production of citrinin is generally reached when the glucose has decreased to about 1 per cent., and it has been found that if incubation is continued after this point there is a gradual decomposition and loss of citrinin.

The metabolism solution is now filtered and the filtrate acidified by the addition of 100 c.c. of concentrated HCl. There is an immediate production of a yellow turbidity which on standing gradually resolves itself into a yellow micro-crystalline deposit. This is filtered off, washed with water and dried, and consists almost entirely of practically pure citrinin. Citrinin is so very slightly soluble in water that only a very small amount is left in the acidified solution after standing overnight, and attempts to recover this are not worth while. The yields of citrinin obtained from 30 litres of metabolism solution varied between 45 and 60 gm. As thus obtained citrinin is a very light yellow powder which on inhalation gives rise to violent fits of sneezing.

A table, Table I, is attached giving particulars of the various batches of citrinin produced, the various data included being the time of incubation, residual glucose, production of material as estimated by alkaline iodine (see p. 276) and yield of crude product actually recovered from 30 litres of medium in each case.

It will be noted on examination of Table I that with batches 8 and 10 the cultures used were spp. Ad. 95 and Ad. 114 respectively, as mentioned on pp. 269 and 270. These batches were grown with a view to ascertaining definitely that these two strains would produce citrinin when grown under identical conditions with Ad. 23.

Effect of temperature on the yield of citrinin.

While engaged on the collection of sufficient material for the purposes of investigation, it was considered advisable to study the temperature conditions for the optimum yield of citrinin.

An experiment was carried out on test tube scale to ascertain the effect of various temperatures on the production of citrinin from glucose by Ad. 23. The following temperatures were employed:—Room temperature, 24°, 28°, 30°, 32°, 34° and 37° C. After 19 days' incubation, tubes were taken off for analysis and the results are shown in Table II.

From these figures it appears that, in general, there is an optimum temperature range of approximately 28° to 32° C.

TABLE I.—Details of experiments for the preparation of citrinin from glucose.

| Expt. | Period of Incubation. | | Containing Vessel. | Residual Glucose by Polarimeter. | Residual Glucose by Alkaline Iodine. | Difference | Yield per 30 litres. | Organism used. | Remarks. |
|-------|-----------------------|----------|----------------------|----------------------------------|--------------------------------------|--------------------|----------------------|----------------|--|
| | Start. | Finish. | | | | | | | |
| 1 | 30.6.27 | 14.7.27 | Tank, 60 litres | Per cent. 0.712 | Per cent. 0.799 | Per cent. 0.087 | Gm. 4.0 | Ad. 23 | Quality of product poor. Grown at room temperature. |
| 2 | 19.7.27 | 10.8.27 | Tank, 60 litres | 0.497 | 0.545 | 0.048 | 9.5 | Ad. 23 | Grown at room temperature. Owing to poor results with tank, future experiments conducted in flasks plugged with cotton wool. |
| 3 | 3.8.27 | 19.8.27 | Flasks, 30 litres | 0.492 | 0.966 | 0.474 | 45.3 | Ad. 23 | Grown at 28–30° C. |
| 4 | 8.8.27 | 22.8.27 | Flasks, 30 litres | 0.773 | 1.343 | 0.570 | 46.0 | Ad. 23 | Grown at 28° C. |
| 5 | 27.8.27 | 12.9.27 | Flasks, 30 litres | 0.790 | 0.928 | 0.138 | 12.7 | Ad. 23 | Grown at 28° C. Product dark in colour, amorphous. |
| 6 | 2.9.27 | 15.9.27 | Flasks, 30 litres | 1.172 | 1.781 | 0.609 | 41.2 | Ad. 23 | Grown at 28° C. Product lighter and more crystalline. |
| 7 | 15.9.27 | 27.9.27 | Flasks, 30 litres | 1.045 | 1.756 | 0.711 | 40.0 | Ad. 23 | Grown at 28° C. |
| 8 | 19.9.27 | 4.10.27 | Flasks, 30 litres | 0.591 | 0.911 | 0.320 | 15.3 | Ad. 95 | Product dark and somewhat tarry. |
| 9 | 1.10.27 | 13.10.27 | Flasks, 30 litres | 0.954 | 1.589 | 0.635 | 58.0 | Ad. 23 | The best yield so far obtained. |
| 10 | 11.6.29 | 29.6.29 | Flasks, 21.75 litres | 1.04 | 1.38 | 0.34 | 19.3 | Ad. 114 | Product dark and somewhat tarry, cf. experiment 8. |
| 11 | 14.11.27 | 26.11.27 | Flasks, 30 litres | 0.995 | 1.647 | 0.652 | 41.9 | Ad. 23 | |

TABLE II.

| Temperature of Incubation. | Total Glucose by Alkaline Iodine. | Glucose by Polarimeter. | Net Iodine Figure. | Reaction with HCl. | Reaction with excess FeCl ₃ . |
|----------------------------|-----------------------------------|-------------------------|--------------------|-----------------------------------|--|
| | | | | | Comparative Dilution. |
| Room | per cent. 4.118 | per cent. 4.016 | per cent. 0.102 | Slight precipitate after standing | 0 (standard). |
| 24° C. | 2.425 | 2.041 | 0.384 | Medium precipitate ... | 0.7 c.c. |
| 28° C. | 2.161 | 1.656 | 0.505 | Medium precipitate ... | 6.0 c.c. |
| 30° C. | 1.990 | 1.574 | 0.416 | Medium precipitate ... | 6.5 c.c. |
| 32° C. | 1.634 | 0.516 | 1.118 | Medium precipitate ... | 7.0 c.c. |
| 34° C. | 0.878 | 0.775 | 0.103 | Slight opalescence on standing | 3.7 c.c. |
| 37° C. | 1.765 | 1.734 | 0.031 | Slight opalescence on standing | 1.5 c.c. |

Note.—The figures in the last column represent the degrees of dilution to correspond with the depth of colour in an undiluted tube grown at room temperature.

Production of citrinin by P. citrinum Ad. 95.

A number of flasks were prepared containing a medium exactly as used for Ad. 23. These were filled and sterilized in the manner described on p. 270 and after sterilization were sown with an emulsion of spores of *P. citrinum* Ad. 95. The flasks were then incubated in the usual fashion at 28° C., and after the production of citrinin had reached a maximum (*i.e.*, after 15 days) as shown by the difference between the glucose estimated by polarimeter and the glucose estimated by alkaline iodine, the metabolism solution was filtered, acidified as before, and the precipitated product filtered off for purification.

The product in this case, being somewhat dark in colour and apparently not very pure, was placed in a Soxhlet thimble and extracted for some hours with ether. This gave rise to a crop of yellow crystals which, after recrystallising from alcohol, melted with decomposition at 170°–172° C., *cf.* p. 274. On titration 0.1668 gm. of this product required 6.78 c.c. of N/10 NaOH corresponding to a combining weight of 249.6, thus showing that the crystalline substance isolated is the same as that produced by Ad. 23, *cf.* p. 275.

Production of citrinin by P. citrinum Ad. 114.

A similar experiment was carried out using cultures of *P. citrinum* Ad. 114. In this case the production of citrinin had reached a maximum after 18 days. The product, which was yellow brown in colour, was isolated by precipitation with acid as before. A portion of this dried product was extracted with ether in a Soxhlet apparatus and the dried extract was recrystallised from alcohol. Typical yellow prisms were obtained

which gave the characteristic colour reaction with FeCl_3 and melted with decomposition at 170°C . The yield of crude citrinin obtained from 21.75 litres of modified CZAPEK-Dox solution was 14 gm.

Purification of citrinin.

The samples of citrinin obtained as described on p. 271 by simple acidification of the metabolism solution are sufficiently pure for most purposes, but in order to obtain an absolutely pure sample for analysis the crude citrinin was recrystallised several times from boiling absolute alcohol in which it is readily soluble, and from which it separates in golden yellow prismatic needles, which if left undisturbed during crystallisation may attain a length of 1 cm. These crystals are highly refractive.

General properties of citrinin.

Citrinin crystallises from alcohol in golden yellow prismatic needles which are practically insoluble in cold water but dissolve to a very slight extent on boiling and separate on cooling in the form of a fine suspension.

It is readily soluble in aqueous NaOH , or aqueous Na_2CO_3 (with evolution of CO_2), giving rise to an orange yellow solution which on standing changes colour to orange red. It is also soluble in aqueous solutions of sodium acetate giving rise to a pale yellow solution, thus indicating the presence in the molecule of a carboxyl group. It is immediately precipitated on acidification of its aqueous solution in NaOH , Na_2CO_3 or sodium acetate.

It is slightly soluble in hot ether from which solvent it crystallises in needles on evaporation. It is fairly readily soluble in both chloroform and acetone. Alcohol is the most suitable solvent for recrystallising citrinin since it is not very soluble in cold alcohol but is readily soluble on heating.

A neutral aqueous solution of citrinin in sodium hydroxide gives a heavy buff coloured precipitate with ferric chloride which is soluble in excess of ferric chloride, giving rise to a very intense iodine-brown coloured solution. A similar colour reaction is given by ferric chloride with an alcoholic solution of citrinin. It immediately decolorises acid potassium permanganate solution in the cold and on treatment with alkaline iodine is oxidized with production of iodoform.

On heating with soda lime pungent vapours are given off with an odour strongly reminiscent of phenol.

On heating, citrinin melts at 166° – 170°C . with obvious decomposition,

Analysis of citrinin.

A sample of citrinin recrystallised from absolute alcohol, and air dried, does not lose weight on drying at 100°C ., thus indicating the absence of solvent of crystallisation.

A sample of citrinin recrystallised from absolute alcohol and dried at 100° C. gave the following results on combustion :—

TABLE III.

| Weight of Citrinin Analysed. | Weight of CO ₂ . | Weight of H ₂ O. | Percentage Carbon. | Percentage Hydrogen. |
|--|-----------------------------|-----------------------------|--------------------|----------------------|
| Gm. | Gm. | Gm. | | |
| 0·1434 | 0·3271 | 0·0721 | 62·20 | 5·63 |
| 0·1278 | 0·2921 | 0·0638 | 62·32 | 5·58 |
| Theoretical for C ₁₃ H ₁₄ O ₅ | — | — | 62·37 | 5·64 |

Determination of combining weight of citrinin by titration.

0·3941 gm. of dry citrinin was suspended in water and N/10 NaOH added to the first appearance of a permanent pink with phenolphthalein. 15·77 c.c. of N/10 NaOH were required, corresponding to a combining weight of 249·9.

Assuming that citrinin titrates as a monobasic acid, its molecular weight is thus 249·9, or if it is a dibasic acid, 499·8. The molecular weight of C₁₃H₁₄O₅ is 250 so that it appears probable that citrinin has the formula C₁₃H₁₄O₅ with a molecular weight of 250 and titrates to phenolphthalein as a monobasic acid.

Molecular weight of citrinin.

In a determination of the molecular weight by RAST's method, 0·00354 gm. citrinin caused a depression of 9·89° C. in the melting point of 0·03994 gm. camphor. This corresponds to a molecular weight of 356. It was noted, however, that distinct signs of some decomposition or interaction between the citrinin and camphor were visible during the experiment, a fact which would render the above estimation invalid.

Optical activity of citrinin.

Citrinin is lævorotatory in alcoholic solution. 0·4996 gm. of citrinin was dissolved in 100 c.c. of absolute alcohol and polarised in the mercury green light in a 20 cm. tube. The mean of a number of rotations was — 0·4167° corresponding to $[\alpha]_{\text{Hg. green}} = -41·7°$.

0·2543 gm. dissolved in 25·08 c.c. absolute alcohol gave an average rotation of — 0·891° in a 20 cm. tube with the mercury green light. This corresponds to a specific rotation of $[\alpha]_{\text{Hg. green}} = -43·9°$.

Determination of methoxyl groups in citrinin.

An ordinary ZEISEL estimation was carried out in the usual PERKIN form of apparatus.

0.1968 gm. of dry citrinin gave 0.0059 gm. silver iodide thus indicating the absence of any methoxyl groups in the molecule of citrinin.

Action of alkaline iodine on citrinin.

0.2559 gm. of citrinin was dissolved in the theoretical amount of sodium hydroxide and made up to 100 c.c. with water, this representing an approximately M/100 solution of citrinin. Varying amounts of this standard solution were then treated with 50 c.c. of N/10 iodine and to the mixture were added during the space of three minutes 75 c.c. of N/10 NaOH. This mixture was then allowed to stand for one hour, acidified with 10 c.c. of N/1 HCl and the iodine liberated was titrated with N/10 sodium thiosulphate. The results are given in Table IV.

TABLE IV.

| c.c. of Solution of Citrinin used. | c.c. of N/10 Iodine absorbed. | c.c. N/10 Iodine absorbed by Indicated Volume of exactly M/100 citrinin. | c.c. N/10 Iodine absorbed per 10 c.c. M/100 Citrinin. |
|------------------------------------|-------------------------------|--|---|
| 1.0 | 1.15 | 1.13 | 11.27 |
| 2.0 | 1.99 | 1.94 | 9.70 |
| 5.0 | 4.56 | 4.45 | 8.90 |
| 10.0 | 8.45 | 8.25 | 8.25 |
| 15.0 | 11.37 | 11.11 | 7.41 |
| 20.0 | 14.00 | 13.68 | 6.84 |

The figures in the last column thus represent the number of atoms of iodine taken up by one molecule of citrinin. Under the particular conditions of the experiment this figure seems to vary between 7 and 11.

The solution containing the higher concentrations of citrinin contained a yellow precipitate which smelt of iodoform and those with 10 c.c. or more were pink in colour, the intensity of colour being proportional to the concentration of citrinin.

It was definitely established that iodoform is formed by the action of alkaline iodine on citrinin by the following procedure: 1.25 gm. of citrinin were dissolved in 50 c.c. of N/10 NaOH to which were added 3,825 c.c. of water and 2,500 c.c. of N/10 iodine. To this mixture 375 c.c. of N/1 NaOH were added slowly and with constant shaking. The mixture was allowed to stand for about an hour by which time a yellow precipitate had formed and the solution was coloured pale pink. The precipitate was filtered off and recrystallised from acetone. It crystallised in typical yellow hexagonal plates having a melting point of 118°–122° C. and the characteristic odour of iodoform.

Derivatives of citrinin.

Various attempts were made to obtain crystalline derivatives of citrinin and the particulars of the methods adopted are as follows :—

(1) *Acetylation of citrinin.*—An attempt to acetylate citrinin by treatment with acetic anhydride and anhydrous sodium acetate resulted in an oil which could not be crystallised, but an estimation of the hydroxyl content of citrinin was carried out, according to the method of PETERSON and WEST (1927).

0·7795 gm. of citrinin gave an acetyl value corresponding to 3·16 c.c. of N/1 NaOH which corresponds to the acetylation of one hydroxyl group in citrinin under the conditions employed, assuming that acetylcitrinin titrates as a monobasic acid.

(2) *Ethylation of citrinin.*—2 gm. of citrinin were suspended in a little dry ether and were ethylated by the addition of an excess of an ethereal solution of diazoethane. A vigorous reaction occurred immediately, slowing down quickly until in about 10 minutes no further evolution of gas was evident. The mixture, still containing a large excess of diazoethane, was allowed to stand overnight. In the morning the ether and residual diazoethane were evaporated off and the ethylated citrinin dried at 60° C. *in vacuo*.

As thus obtained, ethylated citrinin consisted of a pale yellow syrup insoluble in water or sodium hydroxide and resisted all attempts to crystallise it. It distilled at a pressure of about 1 mm. with a bath temperature of 170°–210° C. The distillate consisted of a pale yellow oil which did not crystallise. As there were obvious signs of decomposition the undistilled material was used for analysis.

A methyl compound, similar in appearance, physical and chemical properties, to the ethyl compound, is produced when citrinin is methylated by means of dimethyl sulphate and sodium hydroxide.

The ethoxyl content of the undistilled ethylated citrinin dried to constant weight at 60° C. *in vacuo*, was determined by a ZEISEL estimation with the results given in Table V.

TABLE V.

| Weight of Substance Analysed. | Weight of Silver Iodide. | Percentage OC ₂ H ₅ . |
|--|-----------------------------|--|
| Gm. | Gm. | |
| 0·2460 | 0·3846 | 29·96 |
| 0·1792 | 0·2822 | 30·17 |
| 0·2265 | 0·3528 | 29·85 |
| Theoretical for C ₁₅ H ₁₂ O ₃ (OC ₂ H ₅) ₂ | — | 29·41 |

The product is thus a diethyl compound, and since it is insoluble in sodium hydroxide it appears that the carboxyl group has been esterified and that the product is therefore an ethyl ester of *O*-mono-ethylcitrinin, $C_{12}H_{12}O_2 \cdot (OC_2H_5) \cdot (COOC_2H_5)$.

3. *Reduction of citrinin*.—When citrinin is reduced by means of nascent hydrogen it loses its orange yellow colour and gives rise to a colourless product.

5 gm. of citrinin were dissolved in methyl alcohol, 1–2 c.c. of glacial acetic acid added and then an excess of zinc dust was added in small amounts, the mixture being kept cold. The yellow colour of the citrinin quickly disappeared and when the liquid was quite colourless it was diluted with water, acidified with a little dilute sulphuric acid and shaken out with ether. Although the reduction product is colourless it absorbs oxygen so quickly that the ethereal solution is distinctly yellow in colour in a few minutes. Owing to the instability of the reduction compound an analysis was not carried out on it but it showed the following properties: Reduced citrinin when freshly prepared is a white crystalline product which melts at about 240° C. Its alcoholic solution gives a bright blue colour with a trace of ferric chloride, but with excess of ferric chloride an iodine brown colour is obtained exactly like that given by citrinin itself, reduced citrinin being apparently readily oxidized by ferric chloride to citrinin.

The formula for reduced citrinin can be inferred from:—

4. *Diacetyl compound of reduced citrinin*.—5 gm. of crude citrinin were dissolved by warming in 30 c.c. of methyl alcohol and 2 c.c. of glacial acetic acid were added. To this, zinc dust was added in small portions with shaking until the mixture was nearly colourless and hydrogen began to be evolved vigorously. The temperature was maintained at about 40° – 50° C. during the reduction which took about 10 minutes. The mixture was transferred to a separating funnel, acidified with H_2SO_4 and extracted with ether. The white precipitate which forms on acidifying is immediately soluble in the ether. The ether was filtered and quickly evaporated and the residue in the flask dried overnight *in vacuo* over H_2SO_4 and KOH.

Next day 15 c.c. of acetic anhydride and 7.5 c.c. of pyridine were added, and the acetylation effected by leaving for 24 hours at 37° C. The mixture was diluted with water, filtered from a small amount of insoluble material, and the acetyl derivative precipitated from the clear filtrate by acidification with dilute H_2SO_4 . The acetyl derivative was purified by redissolving in aqueous sodium acetate solution, decolorising with blood charcoal and acidifying the clear colourless filtrate. It was finally washed thoroughly and dried for several days *in vacuo* over P_2O_5 .

This material was analysed as follows:—

A weighed quantity was dissolved in alcohol and titrated with N/5 sodium hydroxide to phenolphthalein (column 2, Table VI). An excess of 100 c.c. of N/5 sodium hydroxide was now added and the mixture hydrolysed by boiling for three hours in an atmosphere of nitrogen. After cooling, the residual excess of N/5 sodium hydroxide was titrated with N/5 H_2SO_4 , the difference representing the amount of acid formed on hydrolysis being given in column 4, Table VI. Finally the acetic acid formed during hydrolysis

was estimated by exhaustive distillation *in vacuo*. The combined distillates were titrated with N/10 sodium hydroxide and this figure was corrected for a small amount of formic acid formed during hydrolysis, the amount of which was estimated by a modification of FINCKE's method (see Part VIII). The corrected figures are given in Table VI, column 6.

TABLE VI.

| Weight of Material Analysed. | Initial Titration. | | Acidity formed on Hydrolysis. | | Acetic Acid found on Hydrolysis. | |
|------------------------------|--------------------|-------------|-------------------------------|-------------|----------------------------------|-------------|
| | Found. | Calculated. | Found. | Calculated. | Found. | Calculated. |
| Gm. | c.c. N/5. | c.c. N/5. | c.c. N/5. | c.c. N/5. | c.c. N/10. | c.c. N/10. |
| 0.6084 | 9.14 | 9.06 | 17.84 | 18.11 | 36.04 | 36.22 |
| 0.6110 | 9.06 | 9.09 | 17.31 | 18.19 | 36.05 | 36.37 |

Assuming that the acetyl compound has the formula $C_{12}H_{13}O \begin{matrix} (O.CO.CH_3)_2 \\ COOH \end{matrix}$ the following calculations have been made :—

- (a) Initial titration value Table VI, column 3.
 (b) Acidity formed on hydrolysis... .. Table VI, column 5.
 (c) Acetic acid formed on hydrolysis Table VI, column 7.

The agreement between the calculated values and those found by analysis is very good.

The diacetyl derivative of reduced citrinin is not easy to recrystallise from any organic solvent. Three different samples were prepared for combustion :—

- (a) Ex acetone ; (b) ex acetone and ether ; (c) ex methyl alcohol.

The first two samples (a and b) contained solvent of crystallisation which is lost on drying at 100° C. The product recrystallised from acetone separated in colourless prisms which give no colour with ferric chloride, and are readily soluble in aqueous solutions of sodium hydroxide, sodium carbonate, or sodium acetate. On heating, this product begins to darken in colour about 200° C., finally becoming very dark and melting with decomposition at 322°–323° C.

Combustion results obtained on each of the above three samples after drying to constant weight *in vacuo* over P_2O_5 at 50° C. are given in Table VII (SCHOELLER, Berlin).

The results given in Tables VI and VII indicate that while citrinin ($C_{13}H_{14}O_5$) only contains one hydroxyl group, since it gives a mono-acetyl derivative (p. 277) and a mono-ethyl ether of the ethylester (p. 277), reduced citrinin contains two hydroxyl groups since it forms a diacetyl derivative. The formation of an additional hydroxyl group on

TABLE VII.

| Sample Analysed. | Weight of Substance Analysed. | Weight of CO ₂ . | Weight of H ₂ O. | Per cent. C. | Per cent. H. |
|--|-------------------------------|-----------------------------|-----------------------------|--------------|--------------|
| | Mgm. | Mgm. | Mgm. | | |
| (a) Ex acetone | 4.773 | 10.485 | 2.62 | 59.90 | 6.14 |
| (b) Ex ether-acetone | 4.815 | 10.610 | 2.65 | 60.10 | 6.16 |
| (c) Ex methyl alcohol | 4.829 | 10.650 | 2.57 | 60.16 | 5.96 |
| Theoretical for diacetyl dihydrocitrinin C ₁₂ H ₁₂ O ₃ (COCH ₃) ₂ COOH ... | | | | 60.69 | 6.00 |
| Theoretical for diacetyl citrinin C ₁₂ H ₁₁ O ₃ (COCH ₃) ₂ COOH | | | | 61.05 | 5.43 |

reduction is further indicated by the fact that while citrinin gives an iodine brown colour with FeCl₃, reduced citrinin gives an initial blue colour becoming iodine brown on addition of excess of FeCl₃.

(5) *Bromine compound of citrinin*.—A bromine compound was obtained by treating citrinin with bromine in glacial acetic acid. This gave rise to a yellow crystalline compound, M.Pt. 121° C., which decomposed readily on treatment with water, with the formation of hydrobromic acid.

(6) *Distillation of citrinin with zinc dust*.—An attempt was made to obtain the basal aromatic nucleus contained in the molecule of citrinin by distillation with zinc dust, but no product could be isolated except a small quantity of tarry distillate with a strong phenolic odour.

This phenolic odour is also noticed when citrinin is heated with soda lime.

(7) *Phenylhydrazide of citrinin*.—It was found possible to prepare a crystalline derivative of citrinin by acting on it with phenylhydrazine in acetic acid solution.

5 gm. of citrinin were dissolved in a mixture of 5 c.c. of phenylhydrazine and 10 c.c. of glacial acetic acid. The whole formed an oily mixture on warming slightly. 100 c.c. of water were then added and the mixture heated on a boiling water bath for 15 minutes with constant stirring. On cooling the product separated as a sticky lump which was washed several times by boiling with water and finally purified by crystallising from absolute alcohol. The substance crystallises in pale yellow, flat prisms, not quite plates, and melts at 207° C. with decomposition. One striking property of this substance is the great rapidity with which its solutions in alkali darken on exposure to air, the colour being brownish yellow at the start and rapidly changing through green and dark blue until practically black.

This compound is insoluble in water, in aqueous potassium acetate or bicarbonate solution but dissolves in dilute sodium hydroxide solution. It gives a cherry red colour with FeCl₃ solution, contains nitrogen, and with FeCl₃ and concentrated H₂SO₄ gives a

deep blue colour. The last-mentioned colour reaction is one which is commonly shown by phenylhydrazides.

Nitrogen analyses by DUMAS' method gave 8·44 per cent. and 8·33 per cent. N₂.

A phenylhydrazide or a phenylhydrazone of citrinin would require a nitrogen content of 8·24 per cent. corresponding to C₁₉H₂₀O₄N₂ while a phenylhydrazine salt of citrinin would require a nitrogen content of 7·82 per cent. corresponding to C₁₉H₂₂O₅N₂.

Taking the above figures for the nitrogen content of this compound, and also considering the fact that it is insoluble in potassium acetate solution and that citrinin cannot be shown to exhibit ketonic properties with other ketonic reagents, it appears probable that the derivative obtained is a phenylhydrazide of citrinin.

As little success had been obtained in attempts to make derivatives of citrinin the breakdown products of citrinin were now investigated.

DECOMPOSITION PRODUCTS OF CITRININ.

A. BY ACID HYDROLYSIS.

It was observed on a small scale that when citrinin is boiled with dilute sulphuric acid it loses its colour and obvious decomposition takes place.

An acid hydrolysis on quantitative lines was therefore carried out as follows :—

1·1036 gm. of citrinin were boiled with 50 c.c. of 2N.H₂SO₄ for four hours by which time the citrinin had gone completely into solution. The hydrolysis was carried out in an atmosphere of nitrogen, freed from oxygen and carbon dioxide, and the gaseous products were passed up a reflux condenser and bubbled through a measured quantity of barium hydroxide. CO₂ was obviously evolved and was estimated by titration of the barium hydroxide.

CO₂ equivalent to 16·97 c.c. of N/2 HCl was evolved, corresponding to 16·9 per cent. of CO₂. Assuming that one molecule of citrinin, C₁₃H₁₄O₅, loses one molecule of CO₂, 17·6 per cent. of CO₂ would be theoretically evolved.

The residual hydrolysis solution was now diluted and distilled exhaustively *in vacuo* until the volatile acids present had been completely removed. The volatile acid distilled was titrated with N/10 NaOH, 43·40 c.c. being required for neutralisation. Assuming that one molecule of citrinin, C₁₃H₁₄O₅, gives rise on acid hydrolysis to one molecule of volatile acid, 1·1036 gm. of citrinin would require 44·14 c.c. of N/10 NaOH to neutralise the volatile acid produced.

Hence the inference from the above figures is that on hydrolysing with 2N.H₂SO₄ one molecule of citrinin gives rise to one molecule of CO₂ and one molecule of a volatile acid.

Identification of the volatile acid formed by acid hydrolysis of citrinin.

5 gm. of citrinin were hydrolysed by boiling with 250 c.c. of 2N.H₂SO₄ for four hours, the hydrolysis mixture was evaporated *in vacuo* to complete removal of the volatile

acid, and the distillate neutralised with sodium hydroxide. This solution of the sodium salt of the volatile acid was evaporated to dryness and qualitative tests on a portion of this showed the following reactions :—

- (1) Decolorisation of acid permanganate solution.
- (2) Reduction of aqueous silver nitrate.
- (3) Reduction of mercuric chloride solution on boiling, with the formation of mercurous chloride.

This indicated the presence of formic acid.

The presence of formic acid was confirmed by the preparation of lead formate. A portion of the sodium salt was dissolved in water, acidified with phosphoric acid, and distilled *in vacuo*. The acid distillate was boiled with lead carbonate, filtered while still hot, and the clear filtrate evaporated. On cooling, lead formate crystallised in typical long needles which were recrystallised for analysis.

0.2651 gm. gave 0.2851 gm. of lead chromate corresponding to a percentage of lead in the above lead salt of 68.9 per cent. (Theoretical percentage of lead in lead formate = 69.7 per cent.)

The question now arose as to whether formic acid is the only volatile acid formed by the acid hydrolysis of citrinin. It was definitely proved that it is really so by the following means: The acids from a portion of the sodium salts were recovered by dissolving the latter in water, acidifying with phosphoric acid and distilling *in vacuo*. The aqueous distillate was made up to 500 c.c. and the following estimations carried out on this solution :—

- (1) The formic acid content was estimated specifically by a modification of FINCKE's method (see Part VIII). The amount of formic acid present by this method in 500 c.c. was 0.360 gm.
- (2) The *total* acid content was estimated by titration with sodium hydroxide to phenolphthalein and gave a total acidity calculated as formic acid of 0.374 gm.

It is thus evident that formic acid is the only volatile acid product of the acid hydrolysis of citrinin.

Investigation of the products of acid hydrolysis of citrinin other than CO₂ and formic acid.

5 gm. of citrinin were hydrolysed by boiling for four hours with 100 c.c. of 2N.H₂SO₄. The solution was cooled, filtered from a little tar that was formed and polarised. The rotation in a 10 cm. tube was -1.35° . The rotation for a 5 per cent. solution of citrinin under these conditions is approximately -2.2° .

The hydrolysis solution was now exhaustively extracted with ether. After extraction the hydrolysis solution had no longer any optical activity. The ether solution on

evaporation smelt strongly of a volatile acid (presumably formic acid) and was dried overnight *in vacuo* over potassium hydroxide. An orange mass was left containing crystals and weighing 4.16 gm.

This orange mass and a further quantity of material obtained from a second hydrolysis of 5 gm. of citrinin were fractionally crystallised from chloroform giving rise to two different crystalline products.

Product A soluble in hot chloroform and crystallised from this solvent.

Product B separated from Product A by its slight solubility in chloroform and crystallised from ethyl acetate.

Product A.

This is a white crystalline solid crystallising in plates which melt at 128°–130° C. after drying to constant weight at 100° C. As originally prepared this compound contains about 30 per cent. by weight of CHCl_3 as solvent of crystallisation which is lost on drying. It is soluble in water and the aqueous solution gives a blue colour with ferric chloride. The substance is lævorotatory. 0.2541 gm. dissolved in 25.08 c.c. of absolute alcohol gave a negative rotation of 0.885° in a 20 cm. tube with mercury green light corresponding to a rotation of $[\alpha]_{\text{Hg. green}} = -43.7^\circ$. Its solution in alcohol is practically neutral, 0.2037 gm. requiring 0.87 c.c. of N/10 NaOH, using phenolphthalein as indicator.

On drying the original material, 0.3405 gm. lost 0.1132 gm. in weight corresponding to a loss in weight of 33.25 per cent. In a repeat experiment on a different sample 1.1249 gm. lost 0.3126 gm. corresponding to a loss of 27.79 per cent. (Theory for $\text{C}_{11}\text{H}_{16}\text{O}_3 + 1 \text{ mol. CHCl}_3 = 37.78 \text{ per cent.}$)

TABLE VIII.—Analysis of Product A dried to constant weight at 100° C.

| Weight of Substance Analysed. | Weight of CO_2 . | Weight of H_2O . | Percentage C. | Percentage H. |
|---|---------------------------|----------------------------------|---------------|---------------|
| Gm. | Gm. | Gm. | | |
| 0.1236 | 0.3024 | 0.0912 | 66.72 | 8.27 |
| 0.1368 | 0.3352 | 0.1004 | 66.82 | 8.21 |
| Theory for $\text{C}_{11}\text{H}_{16}\text{O}_3$ | — | — | 67.31 | 8.22 |

In a ZEISEL estimation, 0.1479 gm. gave 0.0041 gm. of silver iodide indicating that the substance contains no methoxyl groups. In a molecular weight estimation by the improved RAST method 0.00433 gm. depressed the freezing point of 0.03648 gm. of camphor by 23.35° C. corresponding to a molecular weight of 202. (Theory for $\text{C}_{11}\text{H}_{16}\text{O}_3 = 196$.) In a quantitative acetylation 0.2614 gm. gave an acetyl value equivalent to 2.37 c.c. of N/1 NaOH corresponding to an acetyl value of 9.07 c.c. of N/1 NaOH per gm.

1 gm. of $C_{11}H_{16}O_3$ containing two hydroxyl groups would have an acetyl value of 10.20 c.c of N/1 NaOH.

This product therefore appears to have a molecular formula of $C_{11}H_{16}O_3$, contains no methoxyl groups, and has two of the oxygen atoms present as hydroxyl groups which are acetylated by acetic anhydride and pyridine.

Product B.

This product is a white crystalline solid crystallising in prisms from ethyl acetate with a melting point of 169° – 170° C. It is not readily soluble in cold water but is soluble in dilute NaOH. The aqueous solution gives a blue colour with $FeCl_3$ identical with that obtained with Product A. On drying at 100° C. to constant weight 2.0475 gm. lost 0.0021 gm. showing this material contains no solvent of crystallisation. It is optically *inactive*. 0.2545 gm. dissolved in 25.07 c.c. of absolute alcohol gave no rotation in a 20 cm. tube with mercury green light. On titration 0.2041 gm. dissolved in alcohol required 0.96 c.c. of N/10 NaOH, using phenolphthalein as indicator, showing that the substance is practically neutral in reaction.

TABLE IX.—Analysis of Product B dried to constant weight at 100° C.

| Weight of Substance Analysed. | Weight of CO_2 . | Weight of H_2O . | Percentage C. | Percentage H. |
|-------------------------------|--------------------|--------------------|---------------|---------------|
| Gm. | Gm. | Gm. | | |
| 0.1433 | 0.3519 | 0.1067 | 66.98 | 8.33 |
| 0.1151 | 0.2826 | 0.0848 | 66.95 | 8.24 |
| Theory for $C_{11}H_{16}O_3$ | — | — | 67.31 | 8.22 |

In a molecular weight estimation 0.00283 gm. depressed the freezing point of 0.03140 gm. of camphor by 19.07° C., corresponding to a molecular weight of 188. (Theory for $C_{11}H_{16}O_3 = 196$.)

This compound appears to be the optically inactive stereoisomer of product A.

The following table shows the yields of these two materials which are obtained in a typical acid hydrolysis of a batch of crude citrinin as obtained from the metabolism solution by simple acidification.

From the Table X it can be seen that the yield of Product A is eight to nine times that of product B and the course which this reaction takes would appear to be as follows :—

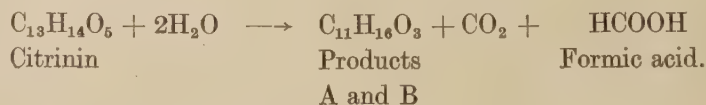


TABLE X.—Distribution of products from a typical acid hydrolysis of citrinin.

| | Weight of Material. | Percentage Yield on original citrinin. |
|--|------------------------|--|
| Citrinin | Gm. 53 | — |
| Tar | 4.16 | 7.9 |
| Total ether extract | 43 | 81.1 |
| Ether extract fractionated as follows :— | | |
| Product A | 26.03 | 49.1 |
| Product B | 3.04 | 5.7 |
| Residual syrup (dark red) | 13.84 | 25.9 |

As the hydrolysis product A, melting point 128° – 130° C., was produced in preponderating amounts it was decided to confine investigations into the nature of the $C_{11}H_{16}O_3$ compounds (*i.e.*, products A and B) to this compound, and the following derivatives were made to obtain what information was possible as to its structure.

Derivatives of acid hydrolysis product A.

(1) *Acetyl compound of product A.*—4 gm. of product A, 4 gm. of sodium acetate and 12 c.c. of acetic anhydride were refluxed for one hour on an oil bath, the mixture diluted with water and the product separated as a colourless sticky oil. This was extracted with ether, the ether solution dried and evaporated. The product separated in small white crystalline plates. Yield 6.4 gm. The acetyl compound crystallises from 40 per cent. alcohol with a melting point of 90° – 91° C., is insoluble in water, and gives no colour with $FeCl_3$ in alcoholic solution.

In an estimation of the acetic acid produced by hydrolysis from this compound 0.4011 gm. gave acetic acid equivalent to 37.29 c.c. of N/10 acid. This weight of diacetyl compound, *i.e.*, $C_{11}H_{14}O(OCOCH_3)_2$ would yield 28.64 c.c. of N/10 acid and of a triacetyl compound, *i.e.*, $C_{11}H_{13}(OCOCH_3)_3$ would yield 37.36 c.c. of N/10 acid. Product A therefore appears to contain three hydroxyl groups which can be acetylated with acetic anhydride and sodium acetate, though only two are acetylated by acetic anhydride and pyridine (see p. 283).

Note on the hydrolysis of the acetyl compound of A.—The non-volatile product from the hydrolysis of the acetyl product of A consists of a whitish solid residue with a melting point of 165° – 167° C. A mixed melting point of this and product B gave 168° – 170° C. Apparently the process of acetylation and deacetylation of Product A causes racemisation of the optically active compound, a fact which confirms the view expressed on p. 284 that Products A and B are optical isomerides.

TABLE XI.—Analysis of acetyl compound of product A.

| Weight of Substance Analysed. | Weight of CO ₂ . | Weight of H ₂ O. | Percentage C. | Percentage H. |
|--|-----------------------------|-----------------------------|---------------|---------------|
| Gm. | Gm. | Gm. | | |
| 0·1323 | 0·3078 | 0·0776 | 63·43 | 6·56 |
| 0·1064 | 0·2459 | 0·0665 | 63·03 | 6·99 |
| 0·1163 | 0·2700 | 0·0705 | 63·31 | 6·78 |
| Theory for C ₁₇ H ₂₂ O ₆ , <i>i.e.</i> , C ₁₁ H ₁₃ (OCOCH ₃) ₃ | | | 63·30 | 6·89 |
| Theory for C ₁₅ H ₂₀ O ₆ , <i>i.e.</i> , C ₁₁ H ₁₄ O(OCOCH ₃) ₂ | | | 64·25 | 7·20 |

In a molecular weight estimation 0·0090 gm. of this acetyl compound depressed the melting point of 0·03171 gm. of camphor by 13·07° C. corresponding to a molecular weight of 278 (Theoretical for C₁₇H₂₂O₆ = 322). This low figure may be due to the product being slightly unstable in molten camphor.

(2) *Dimethyl derivative of product A.*—The hydrolysis product A was methylated as follows: 4 gm. of material were dissolved in 100 c.c. of water and 10 c.c. of dimethyl sulphate added. 10 per cent. NaOH was now slowly added with vigorous shaking to keep the mixture just alkaline, 60 c.c. of 10 per cent. NaOH being used in all. The solution was extracted with ether while still alkaline and the extract obtained was purified by distillation. The weight of extract obtained was 4·10 gm. and after being twice distilled in a high vacuum it gave a boiling point of 136°–138° C. at 1 mm. The product obtained is a colourless, very viscous oil, insoluble in water and dilute NaOH, and gives no ferric chloride reaction in alcoholic solution.

In a ZEISEL estimation 0·2773 gm. and 0·1592 gm. gave 0·5496 gm. and 0·3047 gm. of silver iodide, corresponding respectively to 26·18 per cent. and 25·29 per cent. of methoxyl.

Theory for C₁₁H₁₄O(OCH₃)₂ is 27·68 per cent. methoxyl and for C₁₁H₁₃(OCH₃)₃ is 39·06 per cent. methoxyl.

TABLE XII.—Analysis of dimethyl derivative of product A.

| Weight of Substance Analysed. | Weight of CO ₂ . | Weight of H ₂ O. | Percentage C. | Percentage H. |
|---|-----------------------------|-----------------------------|---------------|---------------|
| Gm. | Gm. | Gm. | | |
| 0·2209 | 0·5629 | 0·1775 | 69·50 | 8·99 |
| 0·1791 | 0·4563 | 0·1428 | 69·48 | 8·92 |
| Theory for C ₁₁ H ₁₄ O(OCH ₃) ₂ | | | 69·58 | 8·99 |

As the methyl derivative produced by this method is only a dimethyl derivative, whereas a triacetyl derivative was obtained by acetylation with acetic anhydride and sodium acetate, a second methylation was carried out on a 1 gm. scale using an ample excess of dimethyl sulphate and the reaction mixture was heated to boiling during the process. The product obtained was an oil boiling at 143° C. at 1.5 mm., which in a methoxyl estimation on 0.1592 gm. gave 0.3179 gm. of silver iodide, corresponding to 26.37 per cent. OCH_3 . This is thus the same product as was obtained by the cold methylation process.

An attempt to recover a product from the hydriodic acid used in the various methoxyl estimations was not successful.

(3) *Monomethyl derivative of product A.*—During one methylation of product A where a large excess of methyl sulphate was not used and the alkalinity was never allowed to become pronounced, a different methylated product was obtained. This was a crystalline solid crystallising from benzene with a melting point of 144° – 147° C. 0.1567 gm. of a purified specimen of this material gave 0.1702 gm. of silver iodide in a ZEISEL estimation, corresponding to a methoxyl content of 14.35 per cent. (Theory for $\text{C}_{11}\text{H}_{15}\text{O}_2(\text{OCH}_3)$ is 14.76 per cent.)

In a molecular weight estimation 0.0034 gm. of this compound caused a depression of 16.64° C. in the melting point of 0.04324 gm. of camphor corresponding to a molecular weight of 201. The theoretical figure for $\text{C}_{11}\text{H}_{15}\text{O}_2.\text{OCH}_3$ is 210.

TABLE XIII.—Analysis of monomethyl derivative of Product A.

| Weight of Substance Analysed. | Weight of CO_2 . | Weight of H_2O . | Percentage C. | Percentage H. |
|--|---------------------------|----------------------------------|---------------|---------------|
| Gm. 0.1213 | Gm. 0.3058 | Gm. 0.0955 | 68.74 | 8.81 |
| Theory for $\text{C}_{11}\text{H}_{15}\text{O}_2(\text{OCH}_3)$ | | | 68.56 | 8.63 |

Acetylated dimethyl compound of product A.—As product A forms a triacetyl, but only a dimethyl, compound an attempt was made to introduce an acetyl group into the dimethyl compound. 1 gm. of dimethyl A, 1.5 gm. of sodium acetate and 5 c.c. of acetic anhydride were refluxed for one hour, cooled, the mixture diluted and the product extracted with ether. The ether extract after drying over H_2SO_4 and KOH weighed 1.13 gm. This product was purified by distillation *in vacuo* giving a viscid colourless liquid boiling at 136° – 138° C. at 1 mm.

In an estimation of the acetic acid formed by hydrolysis of this product, 0.4366 gm. gave a volatile acid equivalent to 15.19 c.c. of N/10 acid. 0.4366 gm. of $\text{C}_{11}\text{H}_{13}(\text{OCH}_3)_2\text{OCOCH}_3$ would require 16.40 c.c. of N/10 acid, on a theoretical basis, for one acetyl

group. As a check on this experiment a similar amount of dimethyl A was subjected to an identical estimation, but only a negligible amount of volatile acid was produced.

This shows definitely that the product A contains three hydroxyl groups, two of which can be methylated or acetylated while the third can be acetylated but not methylated under the conditions employed. As a result of the information obtained from the various derivatives of product A it appears that this compound has the empirical formula $C_{11}H_{16}O_3$, contains an asymmetric carbon atom, and three hydroxyl groups, one of which appears to be alcoholic in nature, while the other two are phenolic.

(B) DEGRADATION PRODUCTS OF PRODUCT A.

As the most favourable method of obtaining information as to the structure of citrinin appeared to be a study of the breakdown products of product A, a considerable quantity of this material was prepared.

The methods of dealing with this substance can be divided into three groups :—

- (1) Direct oxidation, reduction, etc., of product A.
- (2) Oxidation products obtained from dimethyl A.
- (3) Products obtained by fusion of product A with potassium hydroxide.

(1) Attempts were made to obtain oxidation products of Product A by various methods including acid potassium permanganate, alkaline perhydrol, etc., but none of these methods gave rise to a product which could be isolated in a pure state.

Zinc and sulphuric acid at 100° C. have apparently no action on product A, which can be recovered unchanged from the reaction mixture.

An attempt was also made to break down product A by boiling for three hours with 30 per cent. sodium hydroxide, but this process also has no action on the material.

(2) A quantity of dimethyl A was prepared and oxidized by the method recommended by LUFF, PERKIN and ROBINSON (1910) for the production of asaronic acid.

7.5 gm. of purified dimethyl A were placed in a flask (1,500 c.c.) with about 200 c.c. of warm water and arranged on a boiling water bath under a reflux condenser. A 5 per cent. solution of alkaline permanganate was added slowly to the point where the disappearance of the pink colour took more than three minutes. This required about 800 c.c. of permanganate solution. The whole was allowed to cool slightly, filtered from precipitated MnO_2 and the filtrate acidified with hydrochloric acid until just acid to congo red paper. The acidified liquor was now evaporated to dryness, the residue mixed with kieselguhr and the whole extracted in a Soxhlet apparatus with ether. This gave rise to about 5.5 gm. of crude product. The extract was now treated with 50–60 c.c. of water and carefully washed to remove all oxalic acid. The water insoluble portion was then dried (weight = 2.20 gm.), and purified by distillation in a high vacuum. An oily material was obtained which distils at 180°–190° C. at 0.5 mm. and on cooling

sets to a hard transparent glassy material which on treatment with ether separates as a white prismatic crystalline substance with melting point 122° – 124° C.

These crystals are insoluble in *cold* dilute NaOH but are very soluble in chloroform. Quantity obtained, 0.39 gm.

On analysis this product gave the following results:—

TABLE XIV.

| Weight of Substance Analysed. | Weight of CO ₂ . | Weight of H ₂ O. | Percentage C. | Percentage H. |
|---|-----------------------------|-----------------------------|---------------|---------------|
| Gm. 0.1194 | Gm. 0.2894 | Gm. 0.0728 | 66.10 | 6.82 |
| Theory for C ₁₃ H ₁₆ O ₄ | | | 66.07 | 6.83 |

In a ZEISEL estimation 0.1075 gm. gave 0.2098 gm. of silver iodide corresponding to a methoxyl content of 25.77 per cent. Two methoxyl groups in C₁₃H₁₆O₄ would require 26.28 per cent. methoxyl.

In a molecular weight estimation by the RAST method, 0.00284 gm. caused a depression of 13.88° C. in the melting point of 0.03432 gm. of camphor. This figure corresponds to a molecular weight of 237 while the molecular weight of C₁₃H₁₆O₄ is 236.

To test the possibility of this material possessing a lactone or anhydride structure, a small quantity of it was boiled with very dilute NaOH for one hour, cooled, and the mixture titrated to neutrality using phenolphthalein as indicator. The amount of NaOH used in this hydrolysis by 0.0719 gm. of material was 2.89 c.c. of N/10 NaOH. This amount of oxidation product, C₁₃H₁₆O₄, would require 3.05 c.c. of N/10 NaOH to neutralise an opened lactone structure and 6.10 c.c. to neutralise an anhydride structure. The product is therefore probably of the lactone type.

As far as can be assumed from the foregoing facts it appears that the oxidation product obtained by the action of alkaline permanganate on dimethyl A has the empirical formula C₁₃H₁₆O₄, contains two methoxyl groups, and has probably a lactone structure.

(3) *Products obtained by fusing product A with potassium hydroxide.*—As already noted, p. 288, product A is resistant to the action of boiling aqueous 30 per cent. alkali. Accordingly 4 gm. of A were fused in a nickel crucible with 15 gm. of potassium hydroxide and 3 c.c. of water. The crucible was heated in a bath of fusible metal maintained at a temperature of 300° C. for $1\frac{1}{2}$ hours. The mass became semi-fluid, darkened in colour and swelled, with apparent evolution of gas. At the close of the heating period the mixture was allowed to cool, dissolved in about 100 c.c. of cold water, and this solution saturated with CO₂ gas. This caused the precipitation of a crystalline phenolic

body, which was filtered off and washed, first with saturated potassium bicarbonate solution and then with water. Finally the filtrate was extracted with ether to remove the last traces of this product. The yield obtained was 2.9 gm. and on purification by sublimation the product melted at 97°–99° C.

Investigation of phenolic body produced by potash fusion of product A.—This compound is a white crystalline solid which crystallises from water in prisms, from benzene and petrol ether in plates, and sublimes as small polyhedric crystals. It is not readily soluble in cold water nor in an aqueous solution of potassium bicarbonate but dissolves readily in sodium hydroxide solution. Dilute aqueous solutions give a blue colour with a trace of ferric chloride. In dilute alkaline solution it absorbs oxygen from the air and develops a reddish brown colour on standing.

An aqueous solution of this material when titrated with alkali to phenolphthalein has no appreciable titratable value, 0.1045 gm. requiring 0.12 c.c. of N/10 NaOH. When tested for optical activity 0.2514 gm. dissolved in 25 c.c. of water gave a rotation of +0.003° in a 20 cm. tube using mercury green light, the substance therefore being optically inactive.

In a molecular weight estimation 0.00283 gm. of this product caused a depression in the freezing point of 0.03255 gm. of camphor of 24.19° C. corresponding to a molecular weight of 143. A compound of the formula $C_9H_{12}O_2$ would have a molecular weight of 152.

On analysis the substance gave the following figures :—

TABLE XV.

| Weight of Substance Analysed. | Weight of CO ₂ . | Weight of H ₂ O. | Percentage C. | Percentage H. |
|-------------------------------|-----------------------------|-----------------------------|----------------|---------------|
| Gm. 0.1440 0.1284 | Gm. 0.3759 0.3344 | Gm. 0.1048 0.0920 | 71.17 71.00 | 8.14 8.02 |
| Theory for $C_9H_{12}O_2$ | | | 71.00 | 7.96 |

Derivatives of the phenol obtained by potash fusion of product A.

(1) *Acetyl derivative.*—0.5 gm. of the phenol was refluxed for 30 minutes with 0.75 gm. of anhydrous sodium acetate and 2 c.c. of acetic anhydride. On allowing the mixture to cool, diluting with water, and extracting the solution with ether, a syrupy product was obtained which could not be readily purified.

(2) *Dimethyl derivative.*—4 gm. of the phenol were treated with 15 c.c. of dimethyl sulphate and 10 per cent. NaOH added to keep the reaction just alkaline. As the reaction proceeded the rate of methylation was maintained by warming the reaction

mixture to 50° C. After adding 60 c.c. of 10 per cent. NaOH the reaction appeared to be almost complete, a slight excess of NaOH was added and the cooled mixture extracted with ether. The ether extract was washed with a little NaOH solution and then with water to ensure the removal of any unchanged or partially methylated material.

The yield of product obtained by this method is about 4 gm., and it is in the form of a colourless oil which gives no ferric chloride reaction, is insoluble in NaOH, and distils at 89°–91° C. at 1 mm. In a ZEISEL estimation 0.1567 gm. gave 0.3531 gm. of silver iodide, corresponding to a methoxyl content of 29.76 per cent. A monomethyl derivative of the phenol, $C_9H_{11}O \cdot (OCH_3)$ would contain 18.68 per cent. methoxyl and a dimethyl derivative, $C_9H_{10}(OCH_3)_2$, 34.42 per cent. methoxyl. The product in this case is apparently a dimethyl derivative.

(3) *Monomethyl derivative*.—If a sufficient excess of methyl sulphate and sodium hydroxide is not employed a monomethyl derivative of the phenol can be obtained which boils at 100°–120° C. at 1 mm. and crystallises from petrol ether with a melting point of 63°–65° C. This product is soluble in NaOH and is reprecipitated by CO_2 .

In a ZEISEL estimation, 0.1875 gm. gave 0.2619 gm. of silver iodide, corresponding to a methoxyl content of 18.45 per cent. Theory for $C_9H_{11}O \cdot OCH_3$ is 18.68 per cent.

From the foregoing results it appears that the product obtained by the potash fusion of product A is a phenolic body with the empirical formula of $C_9H_{12}O_2$ and contains two hydroxyl groups.

This formula would be satisfied by either a propyldihydroxybenzene, a methylethyl-dihydroxybenzene, or a trimethylhydroxybenzene. The properties of this compound, however, do not appear to agree with any such compounds which are described in the literature.

(C) OXIDATION PRODUCTS OF THE DIMETHYLPHENOL, $C_9H_{10}(OCH_3)_2$.

As the products obtained by the oxidation of dimethyl A and by the potash fusion of product A are compounds which do not appear to have been described in the literature, it was decided to attempt the preparation of some oxidation product of the methylated phenol from the potash fusion, with a view to obtaining something of the nature of a substituted dimethoxybenzoic acid. With this purpose in view a quantity of the dimethylphenol was prepared as already described on p. 289.

The method which was adopted for this oxidation was the same one as that described on p. 288 for the oxidation of dimethyl A. 11.3 gm. of the dimethylphenol, $C_9H_{10}(OCH_3)_2$ were suspended in 200 c.c. of water, heated to 100° C. and 1,000 c.c. of 5 per cent. alkaline permanganate added gradually in 25 c.c. portions. The solution was then allowed to cool, filtered from MnO_2 and acidified to congo red. The acidified solution was now evaporated *in vacuo* to a small bulk, the concentrated liquor made alkaline and extracted with ether to remove any unchanged material. The ether extract containing the unchanged material was combined with an ether extract of the

precipitated MnO_2 , and an ether extract of the condensed distillate from the evaporation, thus ensuring the complete recovery of all unchanged material, which in this case amounted to 5.05 gm. This unchanged material was now subjected to an identical oxidation process on a suitable scale and this repetition was carried on until all the available material had been converted into an alkali soluble oxidation product.

The combined oxidation solutions were acidified with HCl , evaporated to dryness, and the dry residue extracted with ether in a Soxhlet apparatus. The ether extract was evaporated and the residue fractionally crystallised from water. By this means four fractions were finally obtained :—

- (1) Oxalic acid.
- (2) A product melting at $97^\circ\text{--}99^\circ\text{C}$.
- (3) A product melting at $142^\circ\text{--}146^\circ\text{C}$.
- (4) An oily residue.

Oxidation product melting at $97^\circ\text{--}99^\circ\text{C}$.

This product crystallises out of water in white prisms melting at $97^\circ\text{--}99^\circ\text{C}$. without decomposition. It gives no colour with ferric chloride and is strongly acid in reaction. Combustion and ZEISEL results (SCHOELLER, Berlin) correspond to the empirical formula $\text{C}_{11}\text{H}_{14}\text{O}_4$, *i.e.*, $\text{C}_8\text{H}_7(\text{OCH}_3)_2\text{COOH}$. These results are given in Tables XVI and XVII.

TABLE XVI.

| Weight of Substance Analysed. | Weight of CO_2 . | Weight of H_2O . | Percentage C. | Percentage H. |
|--|---------------------------|----------------------------------|---------------|---------------|
| Mgm. | Mgm. | Mgm. | | |
| 4.980 | 11.455 | 2.95 | 62.75 | 6.63 |
| 4.730 | 10.880 | 2.83 | 62.76 | 6.70 |
| Theoretical for $\text{C}_{11}\text{H}_{14}\text{O}_4$ | ... | ... | 62.82 | 6.72 |

TABLE XVII.

| Weight of Substance Analysed. | Weight of Silver Iodide. | Percentage OCH_3 . |
|--|--------------------------|-----------------------------|
| Mgm. | Mgm. | |
| 3.314 | 7.330 | 29.24 |
| 3.321 | 7.370 | 29.33 |
| Theoretical for $\text{C}_9\text{H}_8\text{O}_2(\text{OCH}_3)_2$ | ... | 29.53 |

Oxidation product melting at 142°–146° C.

This product crystallises from water in very fine white needles. It gives no colour with ferric chloride and is strongly acid in reaction. Combustion results (SCHOELLER, Berlin) given in Table XVIII, show that it has the empirical formula $C_{10}H_{12}O_4$.

TABLE XVIII.

| Weight of Substance Analysed. | Weight of CO_2 . | Weight of H_2O . | Percentage C. | Percentage H. |
|-----------------------------------|--------------------|--------------------|---------------|---------------|
| Mgm. | Mgm. | Mgm. | | |
| 5.029 | 11.185 | 2.66 | 60.67 | 5.92 |
| 4.960 | 11.040 | 2.65 | 60.67 | 5.98 |
| Theoretical for $C_{10}H_{12}O_4$ | | | 61.19 | 6.17 |

ZEISEL estimations (SCHOELLER, Berlin), given in Table XIX, indicate the presence of two methoxyl groups.

TABLE XIX.

| Weight of Substance. | Weight of Silver Iodide. | Percentage OCH_3 . |
|---------------------------------|--------------------------|----------------------|
| Mgm. | Mgm. | |
| 3.460 | 8.240 | 31.46 |
| 3.420 | 8.080 | 31.21 |
| Theory for $C_8H_6O_2(OCH_3)_2$ | | 31.64 |

0.0254 gm. titrated to phenolphthalein in alcoholic solution required 1.31 c.c. of N/10 NaOH corresponding to a combining weight of 194 (theoretical for $C_{10}H_{12}O_4 = 196$).

This oxidation product is therefore a dimethoxy carboxylic acid of the formula $C_7H_6(OCH_3)_2 \cdot COOH$.

The question of the constitution of citrinin is discussed in Part XV of this series, but the following is a summary of the salient points established in the work already described and having a bearing on this question.

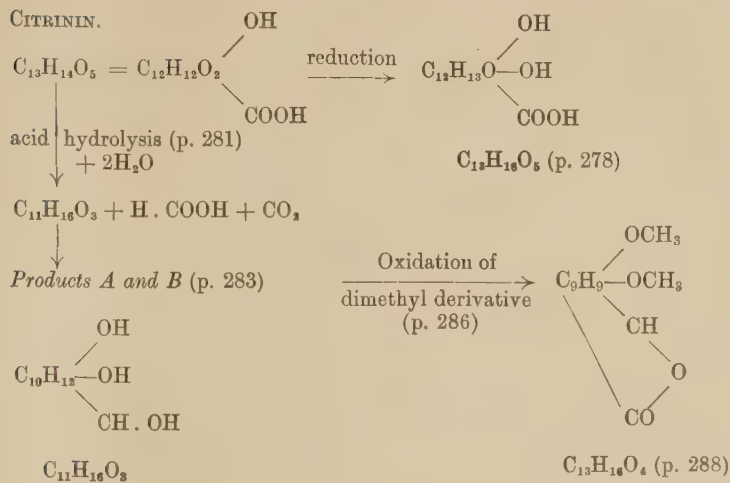
1. Citrinin is a yellow product having the empirical formula $C_{13}H_{14}O_5$.
2. It contains one hydroxyl group and one carboxyl group, since it forms a mono-acetyl compound (p. 277) and an *O*-ethyl ethyl ester (p. 277).
3. It does not contain any methoxyl groups (p. 276) or ketonic groups (p. 280).
4. Since it is optically active it contains an asymmetric carbon atom.

5. The yellow citrinin $C_{13}H_{14}O_5$ containing one hydroxyl group is readily reduced by nascent hydrogen to a colourless dihydroxy compound of the formula $C_{13}H_{16}O_5$ which in its turn is readily re-oxidized to citrinin even by atmospheric oxygen (p. 278).
6. Citrinin is readily hydrolysed by boiling with dilute sulphuric acid, when one molecule of citrinin, $C_{13}H_{14}O_5$, gives rise to one molecule of CO_2 , one molecule of formic acid, and the product $C_{11}H_{16}O_3$, Product A, according to the equation $C_{13}H_{14}O_5 + 2H_2O = CO_2 + HCOOH + C_{11}H_{16}O_3$ (p. 281).
7. Product A ($C_{11}H_{16}O_3$) contains an asymmetric carbon atom and three hydroxyl groups, two of which are phenolic in nature while the third one is alcoholic (p. 283).
8. The dimethyl derivative of product A, $C_{11}H_{14}O (OCH_3)_2$, on oxidation with permanganate gives rise to the product $C_{13}H_{16}O_4$ which is a dimethoxy derivative of an acid lactone (p. 289).
9. Product A ($C_{11}H_{16}O_3$) on fusion with potash gives the phenol $C_9H_{12}O_2$ containing two hydroxyl groups (p. 289).
10. This phenol $C_9H_{12}O_2$ was methylated to the dimethyl compound $C_9H_{10} (OCH_3)_2$ and oxidized with permanganate. Two acids were produced having the empirical formulæ $C_{10}H_{12}O_4$ and $C_{11}H_{14}O_4$. Both are dimethoxy-carboxylic acids, *i.e.*, $C_7H_5 (OCH_3)_2 COOH$ and $C_8H_7 (OCH_3)_2 COOH$ (pp. 291–293).

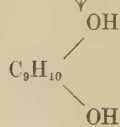
The relationship to citrinin of the various decomposition products is given graphically on p. 295.

Summary.

P. citrinum THOM, when grown on modified CZAPEK-DOX glucose solution, gives rise to a new metabolic product. This is a rich yellow crystalline colouring matter of the empirical formula $C_{13}H_{14}O_5$, to which the name citrinin has been given. A description is given of its preparation, properties, derivatives and breakdown products. It is apparently produced only by *P. citrinum* THOM and seems to be specific for that species.

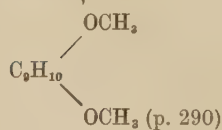


Potash Fusion (p. 289)

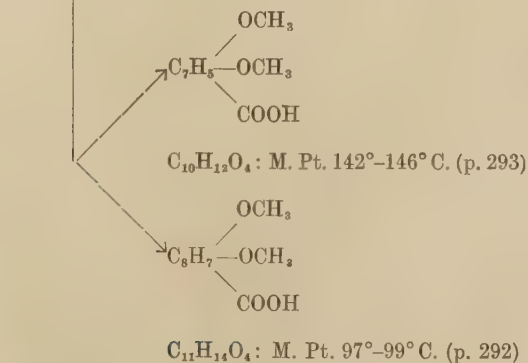


Phenol: $\text{C}_9\text{H}_{10}\text{O}$ (p. 290)

Methylation



Oxidation (p. 291)



*Studies in the Biochemistry of Micro-organisms.*PART XV.—*The Molecular Structure of Citrinin.*

By FREDERICK PHILIP COYNE, HAROLD RAISTRICK and ROBERT ROBINSON, F.R.S.

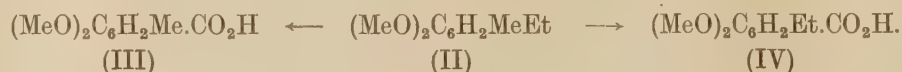
The present communication is, in effect, a discussion of the results obtained in the investigation of citrinin, and described in the preceding memoir (Part XIV); these results, combined with some few further observations, appear to afford a key to the constitution of the substance.

In considering this matter, it is convenient to start with the dihydric phenol, $C_9H_{12}O_2$ (I) (p. 290), which was obtained by fusing either of the isomeric products A or B (p. 283) with potassium hydroxide. This dihydric phenol has the composition of a dihydroxy-propylbenzene, but the relative position of the hydroxyl groups and the arrangement of the alkyl side-chain or chains is unknown. The colour reaction with ferric chloride is, however, in agreement with the view that the substance is a resorcinol derivative, rather less well in harmony with the catechol hypothesis, and is hardly reconcilable with the assumption of a quinol nucleus. The dimethyl ether of I, $C_9H_{10}(OMe)_2$, (II) (see p. 290) gave, on oxidation with potassium permanganate, an acid, $C_7H_5(OMe)_2CO_2H$ (III), m.p. $142^\circ-146^\circ C.$, and another acid, $C_8H_7(OMe)_2CO_2H$ (IV), m.p. $97^\circ-99^\circ C.$ (see pp. 292-293), and examination of these substances has made it clear that I is a resorcinol derivative. Fusion of a mixture of III and IV with potassium hydroxide and recovery of the acid formed gave a product showing a typical resorcylic acid ferric chloride reaction, a violet coloration that was remarkably stable. Further, this product, when heated with phthalic anhydride and a trace of sulphuric acid, gave a phthalein which dissolved in aqueous ammonia to a reddish solution, exhibiting an intense green fluorescence. On strongly heating the product of alkali-fusion of the mixture of III and IV and again fusing with potassium hydroxide and isolating the acidic product, a trace of material was obtained, showing, after condensation with phthalic anhydride, a characteristic fluorescein reaction of resorcinol rather than of alkylresorcinol type. There is thus little doubt that I is a resorcinol derivative, and the formation of the acids III and IV can only be explained on the hypothesis that the substance is a propyl-resorcinol or a methylethylresorcinol—it cannot be a trimethylresorcinol or an isopropyl-resorcinol.

The propylresorcinol possibility is *a priori* unlikely, because it would be necessary to formulate the oxidation of II in accordance with the scheme: $(MeO)_2C_6H_3.CH_2.CH_2.CH_3 \rightarrow (MeO)_2C_6H_3.CH_2.CH_2.CO_2H$ (IV) $\rightarrow (MeO)_2C_6H_3.CH_2.CO_2H$ (III). The only

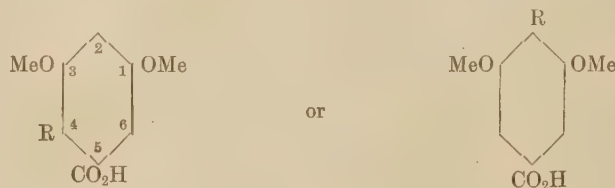
analogies for such graded oxidation of a saturated side-chain in the aromatic series are of very doubtful validity, and it is usually found that permanganate oxidizes fully saturated chains so as to produce derivatives of benzoic acid.

On the view that II is a methylethylresorcinol dimethyl ether, it is possible to represent the formation of III and IV in accordance with the scheme :—



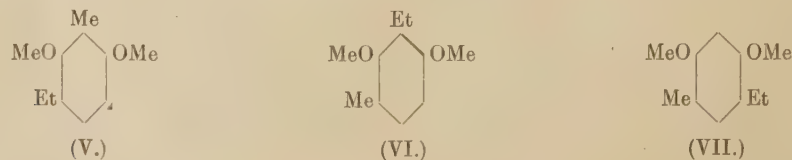
Fortunately, it was possible to show that this scheme does, in fact, represent the degradation, because both III and IV are derivatives of benzoic acid. The direct attachment of the carboxyl group to the nucleus was proved in both cases by applying the CURTIUS reaction to the acids; the amines ultimately obtained were diazotisable and the resulting diazonium salts coupled with β -naphthol to unmistakable azo-compounds. Owing to the small quantities available, the reactions were carried out on a tiny scale, but the results were none the less positive and established this important stage in the argument.

The next point to notice is that neither the methyl nor the ethyl group can be in position 5 in the nucleus, for, if this were so, one of the acids III or IV would be of the form



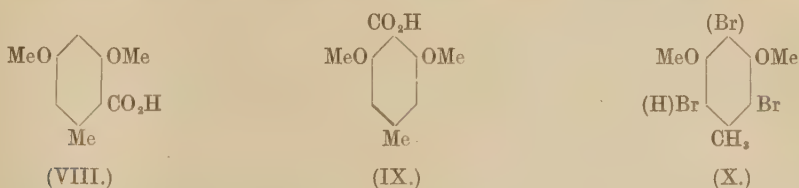
where R = Me or Et. In either case, one of the acids III or IV would be a 3:5-dimethoxybenzoic acid with a free position *o-p.* to methoxyl, and, when heated with sulphuric acid, an intense colour would be developed owing to the formation of an anthrachryson derivative. However, neither III nor IV exhibits such a colour reaction when heated with sulphuric acid.

It follows that II must have one of the three formulæ V, VI or VII.



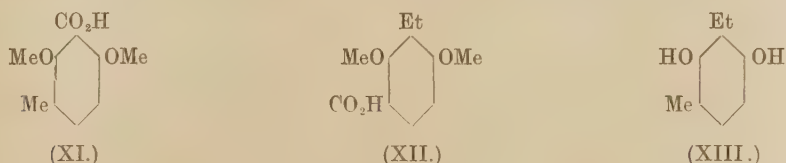
According to formulæ V and VI, the relation of the carboxyl to the methoxyl groups in III and IV will be different, but in VII the relation will be the same and both III and IV would be 2:4-dimethoxybenzoic acids.

In searching for a diagnostic reaction, we were greatly assisted by having in our possession pure specimens of the dimethyl ethers of orsellinic acid (VIII) and of para-orsellinic acid (IX), and a significant divergence in their behaviour was noted when bromine water was added to a cold, dilute solution in aqueous sodium carbonate. This reaction was already known to be capable of distinguishing between somewhat similarly



constituted alkyloxy-benzoic acids (compare JONES and ROBINSON (1917)). Thus, the carboxyl group of veratric acid is displaced by bromine but not that of a methoxy-veratric acid, namely, gallic acid trimethyl ether. The displacement of the carboxyl group occurs, apparently, most easily when methoxyl is situated in the *p*-position to it. VIII gives an immediate precipitate of a neutral di-bromo-derivative (X), whereas IX gave no precipitate under the same conditions.

The substance from VIII crystallised from ethyl alcohol, in which it is sparingly soluble, in colourless prismatic needles, m.p. 168° – 169° C. (Found : Br, 51.2 per cent. $C_9H_{10}O_2Br_2$ requires Br, 51.6 per cent.). It is therefore 2 : 6 (or 2 : 4)-*dibromo-orscinol dimethyl ether*. On testing the acids III and IV in the same way, it was found that no precipitation occurred when bromine water was added to a solution of III in aqueous sodium carbonate; this fact, alone, suggests that III must have the formula XI and excludes VII for the substance II. Clearly, this requires that IV should be represented by the formula XII and should yield a

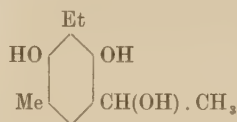


neutral bromo-derivative when bromine is added to its alkaline solution. Actually, this was found to be the case, and the bromo-derivative (probably, 2 : 4-dibromo-6-ethylresorcinol dimethyl ether, but it may also be a mono-bromo-derivative) crystallised from methyl alcohol in colourless needles, but was obtained in such small amount that a fuller description must be reserved.

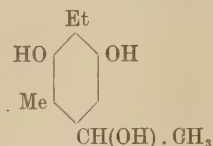
We arrive at the definite conclusion that the dihydric phenol (I) has the constitution XIII, and this is in full agreement with the properties of the substance.

Now, I (XIII) is derived from the products A and B, $C_{11}H_{16}O_3$, by alkali fusion and, from the compositions, the optical activity of product A and chemical analogies (which

exclude attachment of the group, removed by the potash fusion, to the alkyl side-chains), it is only possible to admit one of the formulæ XIV or XV as adequate representations of the constitution of product A (or B).

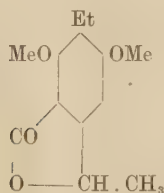


(XIV.)

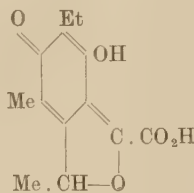


(XV.)

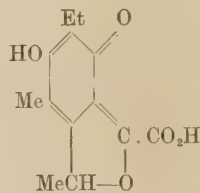
Either of these expressions (XIV or XV) is in harmony with the general character of the substances, $C_{11}H_{16}O_3$, as dihydric phenols containing also an alcoholic hydroxyl group, but XV is to be preferred, (a) because products A and B couple with diazonium salts to azo-compounds and (b) because, on the basis of the formula XV, the *lactone* obtained on oxidation of the dimethyl ether of product A (see Part XIV, p. 289) can be assigned the constitution XVI. No such natural explanation of the results is possible on the basis of XIV for products A and B.



(XVI.)



(XVII.)



(XVIII.)

The substances $C_{11}H_{16}O_3$ (products A and B, XV) are obtained from citrinin, which is a hydroxycarboxylic acid, in accordance with the equation:—



and it appears that this decomposition can only be explained by attributing the formula XVII to citrinin.

The only alternative is the related *o*-quinonoid formula (XVIII), and this is improbable in view of the superior stability of *p*-benzoquinones; moreover, the yellow colour is in good agreement with the *p*-quinone structure.

The constitution XVII serves to illustrate the whole chemical behaviour of the colouring matter, and we are now engaged in synthetical experiments, with the object of confirming the correctness of these deductions. It may be noted that the carbon skeleton of XVII contains two straight chains of six carbon atoms each, joined at their γ -positions by a thirteenth carbon atom.

Studies in the Biochemistry of Micro-organisms.

PART XVI.—*On the production from Glucose by Penicillium Spiculisporum LEHMAN of a new polybasic fatty acid, $C_{17}H_{28}O_6$ (the lactone of γ -hydroxy- $\beta\delta$ -dicarboxypentadecoic acid).*

By PERCIVAL WALTER CLUTTERBUCK, HAROLD RAISTRICK and MARGARET LESLEY RINTOUL.

The acid described in this paper is a metabolic product of *P. spiculisporum* LEHMAN. This species was isolated by S. G. LEHMAN (1919) from the rootlets of apparently healthy cotton plants taken from a field in Anson County, N.C., U.S.A. It derives its name from the fact that "the walls of the ascospores bear minute spines." The culture used was obtained from the American Type Culture Collection and bears their Catalogue No. 1136, THOM and CHURCH Collection 4391.

It was noticed that, when cultures of *P. spiculisporum*, grown in test tubes on the usual CZAPEK-DOX medium, were filtered and acidified, an amorphous insoluble white precipitate was produced. This precipitate may be readily crystallised from boiling water and constitutes the product, the preparation, properties and constitution of which will now be described in detail. This product was shown to be the lactone of γ -hydroxy- $\beta\delta$ -dicarboxypentadecoic acid and for the sake of brevity will be referred to throughout as Substance "A."

It is worthy of mention here, as a further example of the extraordinary specificity of the lower fungi, that Substance "A" is produced only by *P. spiculisporum* and not by any other species either of *Penicillium* or of any genus in our collection.

Preparation of Substance "A."

Thirty litres of the modified CZAPEK-DOX glucose medium containing twenty times the amount of ferrous sulphate given in Part I, p. 7, were made up.

350 c.c. of this medium were distributed in each of a number of 1-litre conical flasks which were plugged with cotton wool, and sterilized by steaming for half an hour on each of three consecutive days. The flasks were then sown with a suspension in distilled water of the spores of *P. spiculisporum* grown in ROUX bottles on beer wort agar (or CZAPEK-DOX agar). The flasks were incubated at 27°–28° C. (and in one case at 37° C.) until the sugar content as determined by the polarimeter was less than 1 per cent. The following table is a typical example of the progress of the growth of the mould.

TABLE I.—Batch of flasks sown 31st March, 1928. Taken off 27th April, 1928.

| Date of Testing. | Incubation Period in Days. | Percentage Glucose by Polarimeter. | Titration in c.c. N/1 NaOH per 250 c.c. Medium. |
|------------------|----------------------------|------------------------------------|---|
| 11.4.28 | 11 | 3.62 | 4.5 |
| 13.4.28 | 13 | 3.41 | 4.6 |
| 16.4.28 | 16 | 2.86 | 4.83 |
| 19.4.28 | 19 | 2.86 | 5.4 |
| 23.4.28 | 23 | 2.25 | 6.23 |
| 26.4.28 | 26 | 1.36 | 7.0 |
| 27.4.28 | 27 | 0.99 | 6.35 |

The metabolism solution was then filtered and acidified with HCl. This gave rise to a voluminous, light, white, amorphous precipitate which was filtered off on a large Buchner funnel and dried. The amount of material obtained from the metabolism solution depends largely on its final acidity and the temperature of incubation. If the final acidity is high and the temperature of incubation low a considerable proportion of Substance "A" is precipitated in the metabolism solution and hence is filtered off with the mycelium, from which it may be readily extracted by means of boiling water. Extraction is repeated two or three times and the boiling water extract slightly acidified with HCl. If the flasks are incubated at 37° C., however, and filtration is rapid a portion of the Substance "A" crystallises out from the filtered metabolism solution on cooling.

A further small amount of Substance "A" may be recovered from the filtered, acidified, metabolism solution either by evaporation *in vacuo* and extraction with ether, or by freezing in the cold room and filtering immediately the frozen solution has been thawed. The amount recovered by either of these methods is small, however, averaging about 5–10 per cent. of the total amount.

Identification of succinic acid as a metabolic product of P. spiculisporum.

The whole of the titratable acidity of the metabolism solution is not accounted for by Substance "A," the greater part being accounted for by the presence of succinic acid. This was proved as follows:—

A quantity of metabolism solution from which Substance "A" had been separated by acidifying with HCl and filtration was evaporated *in vacuo* and extracted with ether. On evaporation of the ether extract a quantity of white crystals remained. A portion of this was sublimed in a high vacuum and had then a melting point of 189° C. which was not changed on mixing with a sample of succinic acid. 0.0484 gm. of the same sublimed material required 8.32 c.c. of N/10 NaOH for neutralisation corresponding

to an equivalent weight of 58.2. (Theoretical for succinic acid = 59.0.) A further amount gave the following results on combustion :—

TABLE II.

| Weight of Material Analysed. | Weight of CO ₂ . | Weight of H ₂ O. | Percentage Carbon. | Percentage Hydrogen. |
|---|-----------------------------|-----------------------------|--------------------|----------------------|
| Gm. 0.1585 | Gm. 0.2366 | Gm. 0.0744 | 40.70 | 5.25 |
| Theoretical for C ₄ H ₆ O ₄ | | | 40.66 | 5.11 |

The yield of Substance "A" obtained under the conditions described is not very large. Details of the various batches prepared are given in the following tables :—

TABLE III.

| Date of Sowing. | Incubation Period in Days. | Temperature of Incubation. | Apparatus. | Volume of CZAPEK-Dox 5 per cent. Glucose Solution used. | Weight of Substance "A" obtained. |
|-----------------|----------------------------|--|--|---|--|
| 3.2.28 | 18 | 28° C. | 44 flasks (750 c.c.) | Litres. 11 | Gm. 10.0 |
| 10.3.28 | 17 | 28° C. | 121 flasks | 30 | 15.3 |
| 31.3.28 | 27 | 28° C. | 169 flasks | 60 | 23.7 |
| 23.4.28 | 35 | Room temp. | 6 enamelled trays in aluminium incubator | 30 | (A further 3.2 gm. separated on freezing) 6.4 |
| | 36 | Room temp. | 6 silica trays in aluminium incubator | 30 | 29.5 |
| 7.5.28 | 22 | 27–28° C. (3 flasks were incubated at 37° C.) | 86 flasks | 30 | 13.6 |
| 5.6.28 | 17 | 37° C. | 108 flasks | 37 | 13.6 |
| 9.6.28 | 17 | 37° C. | 110 flasks | 35 | 12.6 |
| 10.7.28 | 17 | 37° C. | 101 flasks | 35 | 6.2 |
| 10.7.28 | 20 | 37° C. | 108 flasks | 35 | 6.8 |

TABLE IV.—Analysis of average sample from different batches.

| Date of Sowing. | Percentage Glucose by Polarimeter. | Acidity in c.c. N/1 NaOH per 250 c.c. Medium. | Weight of Substance "A" precipitated from 250 c.c. of Acidified Medium Filtered through a Gooch Crucible and dried at 100° C. |
|-------------------|------------------------------------|---|---|
| 3.2.28 | 0.782 | 4.3 c.c. | — |
| 10.3.28 | 0.660 | 2.65 c.c. | — |
| 31.3.28 | 0.994 | 6.35 c.c. | — |
| 23.4.28 | 0.218 | 5.28 c.c. | — |
| (enamelled trays) | | | |
| 23.4.28 | 0.935 | 5.53 c.c. | — |
| (silica trays) | | | |
| | 1.298 | 6.58 c.c. | — |
| | | (Average of flasks incubated at 28° C.) | — |
| 7.5.28 | 0.715 | 9.23 c.c. | — |
| | 0.875 | 1 flask incubated at 37° C. | — |
| | | 8.8 c.c. | — |
| | | 1 flask incubated at 37° C. | — |
| 5.6.28 | 1.024 | 8.53 c.c. | 0.0043 gm. after 6 days. |
| | | | 0.0823 gm. after 13 days. |
| | | | 0.0860 gm. after 15 days. |
| | | | 0.0604 gm. after 17 days. |
| 9.6.28 | 1.051 | 8.12 c.c. | 0.0352 gm. after 9 days. |
| | | | 0.0683 gm. after 17 days. |
| 10.7.28 | 1.576 | 7.4 c.c. | 0.0239 gm. after 15 days. |
| 10.7.28 | 0.825 | 7.5 c.c. | 0.0389 gm. after 17 days. |
| 9.3.29 | 1.009 | 5.59 c.c. | 0.0415 gm. after 20 days. |
| | | | — |

Purification of Substance "A."

The Substance "A" obtained by acidification of the metabolism solution is almost pure and homogeneous. A quite pure product may be obtained by recrystallising from boiling water with the addition of a little animal charcoal. Alternatively Substance "A" may be purified by recrystallising from boiling ethyl ether to which is added boiling light petroleum (boiling point 40°–45° C.) until crystallisation commences. It may also be recrystallised from alcohol, but is so readily soluble in this solvent that this method is not recommended.

Isolation of γ -ketopentadecic acid from the mother liquors remaining after crystallisation of Substance "A."

The ether-light petroleum mother liquors from a long series of purifications of over 100 gm. of crude Substance "A" were combined and the solvent removed. 4.1 gm. of a semi-solid material were obtained. This was boiled with light petroleum several times and the petroleum decanted off, whilst boiling, through a Buchner funnel. 0.5 gm. of crystalline material separated from the petroleum on cooling and melted at 87°–89° C.

After recrystallising twice from boiling petroleum it melted at 92.6°C . and the melting point was unchanged on mixing with the keto-acid prepared by oxidation of Substance "A" with permanganate in acetone solution (see p. 325). It also had the same equivalent, 0.1170 gm. requiring 4.65 c.c. of $\text{N}/10\text{ NaOH}$ corresponding to an equivalent of 251.6 . (Theoretical for $\text{C}_{15}\text{H}_{28}\text{O}_3 = 256$.)

It appears probable, therefore, that, in addition to Substance "A" and succinic acid, γ -ketopentadecic acid is also a metabolic product of *P. spiculiformis* LEHMAN. The possibility cannot be ruled out, however, that γ -ketopentadecic acid is merely an artefact arising from Substance "A" during the purification of the latter substance.

The residue insoluble in light petroleum was dissolved in a little ether and light petroleum added. A small amount (0.3 gm.) of Substance "A" separated but the bulk of the material remained in solution and on removal of solvent proved to be a yellow oil which did not crystallise on standing for a long time in the cold room.

General properties of Substance "A."

When crystallised from boiling water Substance "A" separates in large, shining, white plates which in the liquid appear like fine silky needles. It separates from a mixture of ether and light petroleum in small plates. It is readily soluble in boiling water but almost insoluble in cold water. It is also readily soluble in alcohol and acetone, fairly soluble in ethyl ether, less soluble in chloroform, and almost insoluble in light petroleum. It melts at 145° – 146°C . without decomposition.

Analysis of Substance "A."

A sample recrystallised from water gave on combustion the results given in Table V.

TABLE V.

| Experiment No. | Weight of Substance Analysed. | Weight of CO_2 . | Weight of H_2O . | Percentage Carbon. | Percentage Hydrogen. |
|--|-------------------------------|---------------------------|----------------------------------|--------------------|----------------------|
| 1 | Gm. 0.1319 | Gm. 0.3010 | Gm. 0.1004 | 62.25 | 8.52 |
| 2 | 0.1246 | 0.2823 | 0.0970 | 62.02 | 8.71 |
| Theoretical for $\text{C}_{17}\text{H}_{28}\text{O}_6 \dots \dots \dots$ | | | | 62.15 | 8.59 |

Molecular weight of Substance "A."

(a) *By titration.*— 0.4200 gm. was dissolved in alcohol and titrated to phenolphthalein with $\text{N}/10\text{ NaOH}$. 25.68 c.c. were required corresponding to a combining weight of 163.6 . (Theory for $\text{C}_{17}\text{H}_{28}\text{O}_6$ assuming that this titrates as a dibasic acid = 164 .)

(b) *By analysis of silver salt.*—The silver salt was prepared by precipitation of a solution of the sodium salt with 5 per cent. AgNO_3 solution. A white flocculent precipitate was obtained which was filtered off in the dark, well washed and dried to constant weight at 100°C . 0.2105 gm. of this left 0.0841 gm. of silver on ignition, equivalent to 39.95 per cent. Ag. (Theoretical percentage of Ag in $\text{C}_{17}\text{H}_{26}\text{O}_6\text{Ag}_2 = 39.83$.) This corresponds to a molecular weight of 326.3. (Theoretical for $\text{C}_{17}\text{H}_{26}\text{O}_6 = 328$.)

(c) *By depression of freezing point of camphor.*—(Modified RAST's method. See SMITH and YOUNG, 1927.)

0.00211 gm. of Substance "A" depressed the melting point of 0.02904 gm. of camphor 17.02°C . corresponding to a molecular weight of 169.5. (Theoretical for $\text{C}_{17}\text{H}_{26}\text{O}_6 = 328$.) No explanation is at present forthcoming for the discrepancy between these figures, but it is interesting to note that among the substances which give incorrect molecular weight values by this method are certain well-known polybasic acids.

Optical activity of Substance "A."

The molecule of Substance "A" must contain an asymmetric carbon atom since its solution in alcohol is optically laevorotatory, though curiously enough the solution of its sodium salt in water is dextrorotatory.

(1) *Optical rotation in alcohol.*—1.0335 gm. of Substance "A" dried at 100°C . were dissolved in 25.07 c.c. of absolute alcohol and polarised in a 40 cm. tube using the mercury green light. The average of ten readings = -2.440° corresponding to $[\alpha]_{\text{Hg. green}} = -14.76^\circ$.

(2) *Optical rotation of sodium salt in water.*—0.5011 gm. of Substance "A" was dissolved in the theoretical amount of N/1 NaOH, the solution made up to 25 c.c. and polarised in the mercury green light. In a 40 cm. tube the mean rotation from a number of observations was $+1.201^\circ$ corresponding to $[\alpha]_{\text{Hg. green}} = +14.98^\circ$.

Reactions of an aqueous solution of the sodium salt of Substance "A."

A neutral aqueous solution of the sodium salt of Substance "A" has the following properties:—

- (a) With calcium acetate solution it gives a white precipitate insoluble in warm water.
- (b) With silver nitrate it gives a white precipitate turning red on standing.
- (c) With neutral lead acetate solution it gives a white gelatinous precipitate which when dried is insoluble in ether.
- (d) With copper sulphate solution it gives a blue precipitate.
- (e) It does not reduce BENEDICT'S solution on boiling.
- (f) The aqueous solution froths on shaking, in a manner reminiscent of a soap solution.

Action of acids on Substance "A."

Substance "A" is unaffected by boiling with dilute sulphuric acid, or concentrated nitric acid, from which solvents it may be readily crystallised. Concentrated sulphuric acid dissolves it and the solution does not darken in colour until nearly boiling.

Action of alkalis on Substance "A."

On boiling Substance "A" with dilute sodium hydroxide solution (N/10 or N/1) hydrolysis takes place and this change is dealt with in more detail on p. 308.

Action of halogens on Substance "A."

Substance "A" is not acted on by aqueous alkaline iodine solution, by bromine in glacial acetic acid, or by iodine in carbon tetrachloride (WILS' solution), and hence does not appear to contain a double bond.

Action of alcoholic potassium acetate solution on Substance "A."

When a solution of anhydrous potassium acetate in absolute alcohol is added to a solution of Substance "A" in the same solvent there is no separation of any material, even on standing, indicating that if a potassium salt of Substance "A" is formed under these conditions it must be readily soluble in absolute alcohol.

Acetylation of Substance "A."

(a) *With acetic anhydride and anhydrous sodium acetate.*—A quantity of Substance "A" was heated with acetic anhydride and anhydrous sodium acetate in an oil bath in the usual way. There was immediate darkening in colour, and after five minutes' heating the mixture was dark brown-black in colour, and on dilution with water gave rise to a small amount of a brownish-black oil which could not be purified. The filtrate from this oil was almost colourless but acidification of it gave no precipitate nor did ether extraction give rise to any product. It is somewhat remarkable, in view of the general stability of Substance "A" towards most reagents, that heating with acetic anhydride and anhydrous sodium acetate should decompose it so readily.

(b) *With acetyl chloride.*—On boiling with acetyl chloride Substance "A" gradually dissolved. The excess of acetyl chloride was removed on the water bath and finally *in vacuo* when a colourless oil remained. This oil is readily soluble in absolute alcohol and separates also as an oil from aqueous alcohol. It resisted all attempts to crystallise it.

(c) *With acetic anhydride and pyridine.*—A quantitative acetylation of Substance "A" was carried out by the method described by PETERSON and WEST (1927). About 0.5 gm. of Substance "A" was dissolved in 10 c.c. of a mixture of 8 c.c. of acetic anhydride and 40 c.c. of pure pyridine and incubated at 37° C. for five days in a tube fitted with

a ground glass stopper. Blanks containing 10 c.c. of the pyridine-acetic anhydride mixture were incubated alongside. After five days the contents of each tube were washed out with 200 c.c. of chilled water and titrated with N/1 NaOH.

TABLE VI.

| Weight of Substance Acetylated. | c.c. N/1 NaOH required to Neutralise. | c.c. N/1 NaOH required to Neutralise Blank. | c.c. N/1 NaOH excess of estimation over Blank. | c.c. N/1 NaOH due to original Acidity of Substance "A." | c.c. N/1 NaOH equivalent to Acetic Anhydride used up. | Number of Acetyl groups in one Molecule of Substance "A." |
|---------------------------------|---------------------------------------|---|--|---|---|---|
| Gm. | | | | | | |
| 0.5051 | 36.23 | 33.99 | 2.24 | 3.08 | (3.08-2.24) 0.84 | 0.55 |
| 0.5036 | 36.16 | 33.96 | 2.20 | 3.07 | (3.07-2.20) 0.87 | 0.57 |

HYDROLYSED SUBSTANCE "A."

When Substance "A" is boiled for a short time with an excess of N/1 NaOH, although no apparent change takes place, the product recovered on acidifying the alkaline solution is different in appearance and has a different melting point from Substance "A." This reaction has therefore been examined in some detail.

0.5015 gm. of Substance "A" was boiled with 100 c.c. of N/10 NaOH for eight hours but no CO₂ or volatile acid was produced during the hydrolysis.

Subsequent investigation has shown that on boiling Substance "A" with dilute alkali, the original acidity of Substance "A" is increased by approximately 50 per cent., and that if this substance with the increased acidity is now boiled with dilute acid it reverts to the original Substance "A." The explanation of this appears to be that Substance "A" is a dibasic acid containing two carboxyl groups and a lactone ring. When it is boiled with dilute alkali the lactone ring is opened, giving rise to a hydroxy tricarboxylic acid (subsequently referred to as hydrolysed "A") which in its turn on boiling with dilute acid loses water with the consequent closing of the lactone ring and the re-formation of the dicarboxylic acid, Substance "A." The experimental evidence on which these conclusions are based is given in tabular form in Table VII.

The results given in Table VII were obtained as follows:—

The weight of Substance "A" indicated (column 1) was first titrated with standard sodium hydroxide (column 2), and a measured excess (column 3) of sodium hydroxide was then added and the mixture boiled under a reflux for eight hours. The following day the cooled hydrolysis mixture was titrated back with standard H₂SO₄ (column 5). A measured excess of standard acid (column 7) was now added and the mixture boiled under reflux for about an hour. The cooled solution was titrated with standard sodium

TABLE VII.

| Weight of Substance "A" Hydrolysed. | Volume of Sodium Hydroxide needed for Neutralisation. | Excess of Sodium Hydroxide added for Hydrolysis. | Total Volume of Sodium Hydroxide Added. | c.c. Sulphuric Acid needed to Neutralise. | Excess of Acid Produced during Hydrolysis. | Excess of Sulphuric Acid added for Acid Boil. | c.c. Sodium Hydroxide required to Neutralise after Acid Boil. | Loss in Acidity on Acid Boil. |
|-------------------------------------|---|--|---|---|--|---|---|-------------------------------------|
| Gm. 0.4789 | 29.26 c.c. N/10 | 50.02 c.c. N/10 | 79.28 c.c. N/10 | 37.97 c.c. N/10 | 50.02-37.97 = 12.05 c.c. N/10 | 61.61 c.c. N/10 | 49.73 c.c. N/10 | 61.61-49.73 = 11.88 c.c. N/10 |
| 0.4788 | 29.26 c.c. N/10 | 50.08 c.c. N/10 | 79.34 c.c. N/10 | 39.70 c.c. N/10 | 50.08-39.70 = 10.38 c.c. N/10 | 62.86 c.c. N/10 | 50.00 c.c. N/10 | 68.86-50.00 = 12.86 c.c. N/10 |
| 2.5085 | 30.60 c.c. N/2 (calculated) | 69.44 c.c. N/2 | 100.04 c.c. N/2 | 27.54 c.c. N/2 | 69.44-55.08 = 14.36 c.c. N/2 | — | — | — |
| 2.5046 | 15.48 c.c. N/1 | 34.54 c.c. N/1 | 50.02 c.c. N/1 | 27.60 c.c. N/1 | 6.94 c.c. N/1 | — | — | — |

hydroxide (column 8). These figures show fairly conclusively that the increase in acidity on boiling with dilute sodium hydroxide (column 6) is approximately half of the initial acidity (column 2), and that this increase in acidity is lost by subsequent boiling with dilute sulphuric acid (column 9). The alkaline hydrolysis is evidently not quite so complete when N/10 sodium hydroxide is used as when N/2 sodium hydroxide is used for the purpose (Experiment 3).

Isolation of hydrolysis product of Substance "A."

The neutralised solution from Experiment 3 in Table VII was cleared by shaking in the cold with a little Merck's blood charcoal, filtered, and 22.48 c.c. (column 4—column 5) of N/1 H_2SO_4 added to neutralise exactly the sodium hydroxide combined with the hydrolysis product. A stiff gelatinous precipitate was obtained and was extracted with ethyl ether in which it was readily soluble. The ether extract was evaporated to dryness *in vacuo* and the dried residue recrystallised by dissolving in the minimum amount of boiling ethyl ether and adding to this boiling light petroleum. The hydrolysis product readily separated in shining white plates.

Yield = 2.5 gm., from which it is evident that, since this product is homogeneous, there is only one product of hydrolysis and that no deep seated change has taken place during this process.

General properties of hydrolysed Substance "A."

(1) It crystallises from ethyl ether-light petroleum in flat, square plates, the corners of which are cut off.

(2) It melts sharply at 134° – 135° C. On heating above its melting point bubbles of gas are given off which have been shown to be due to loss of water.

(3) The crystals obtained from ether and light petroleum contain solvent of crystallisation which is lost very slowly on exposure to air, but much more rapidly at 100° C.

Determination of combining weight of hydrolysed substance "A" by titration.

It was recognized that if hydrolysed Substance "A" is a hydroxy-tricarboxylic acid and if Substance "A" is the lactone corresponding to it, then considerable difficulty might be experienced in preparing a sample of perfectly pure hydrolysed "A," because of the ease with which hydroxy acids of certain types are known to revert to the lactone. This difficulty was further intensified by the fact that hydrolysed "A" was known to cling tenaciously to the solvent when a mixture of ether-light petroleum, which is the best crystallising agent, was used in its preparation. An attempt to overcome this difficulty by using chloroform as a solvent instead of ether gave the same result since hydrolysed "A" clings to chloroform as tenaciously as to ether. The problem therefore resolved itself into that of finding a method of removing the solvent from hydrolysed "A" without at the same time removing water and hence re-forming Substance "A."

A sample of hydrolysed Substance "A," free from solvent of crystallisation, and containing a minimum of re-formed Substance "A," was finally obtained as follows:—The air-dried sample of hydrolysed Substance "A," whether recrystallised from ether-light petroleum, or from chloroform, was dried at 60° C. at atmospheric pressure. Most of the solvent is driven off in a few hours and subsequent drying only results in a very slight loss in weight.

0.2124 gm. of hydrolysed Substance "A," crystallised from chloroform, and dried at 60° C. for 185 hours, was dissolved in absolute alcohol and titrated with N/10 sodium hydroxide to phenolphthalein. 17.73 c.c. of N/10 sodium hydroxide were required for neutralisation corresponding to a combining weight of 119.8 (combining weight of the tribasic acid $C_{17}H_{30}O_7 = 115.3$). An excess of N/10 sodium hydroxide was now added, the mixture heated at 60° C. for 24 hours, cooled and titrated with N/10 acid. A further 0.67 c.c. of N/10 sodium hydroxide was neutralised during the hydrolysis corresponding to a total amount of 18.40 c.c. of N/10 sodium hydroxide and a *corrected* combining weight of 115.4.

0.1961 gm. of hydrolysed Substance "A" recrystallised from ether-light petroleum, and dried at 60° C. for 164 hours required 16.30 c.c. of N/10 sodium hydroxide for the primary titration, and a further 0.70 c.c. during the secondary hydrolysis, corresponding to an initial combining weight of 120.3 and a *corrected* combining weight of 115.3.

A portion of the same dried sample of hydrolysed Substance "A" gave the following results on micro-combustion (SCHOELLER, Berlin) as indicated in Table VIII.

TABLE VIII.

| Weight of Hydrolysed "A" Analysed. | Weight of CO ₂ . | Weight of H ₂ O. | Percentage Carbon. | Percentage Hydrogen. |
|--|-----------------------------|-----------------------------|-----------------------|-------------------------|
| Mgm. | Mgm. | Mgm. | | |
| 4.821 | 10.430 | 3.70 | 59.02 | 8.59 |
| 4.454 | 9.630 | 3.50 | 58.97 | 8.79 |
| Theoretical for $C_{17}H_{30}O_7$ | ... | ... | 58.96 | 8.67 |

Determination of combining weight of hydrolysed Substance "A" by analysis of the silver salt.

Samples of the silver salt of hydrolysed Substance "A" were prepared by adding silver nitrate solution to the titrated alcoholic solution of hydrolysed Substance "A," filtering off the amorphous silver salt in a subdued light, washing carefully and estimating the silver content by ignition after drying.

Three samples of hydrolysed Substance "A," prepared under different conditions,

were used and the details are given in Table IX. It is evident from these results that the silver salt contained a small amount of the silver salt of Substance "A" and was therefore probably the salt of the equilibrium mixture of hydrolysed Substance "A" and Substance "A."

TABLE IX.—Silver content of silver salt of hydrolysed "A" prepared under different conditions.

| Weight of Silver Salt Ignited. | Weight of Silver. | Percentage of Silver in Silver Salt. | Equivalent calculated on Silver Content. | Remarks. |
|--------------------------------|-------------------|--------------------------------------|--|--|
| Gm. 0·3611 | Gm. 0·1714 | 47·46 | 120·6 | This silver salt was made from a sample of hydrolysed "A," which had been dried in air for five days and had an equivalent of 121·2. |
| 0·3595 | 0·1707 | 47·50 | 120·5 | This sample, crystallised from ether-light petroleum, had been dried for 164 hours at 60° C. and subsequently hydrolysed in aqueous solution at 60° C. for 24 hours with an excess of N/10 sodium hydroxide. |
| 0·3895 | 0·1861 | 47·78 | 119·0 | This sample, crystallised from chloroform, had been dried for 185 hours at 60° C. and subsequently hydrolysed as in previous experiment. |

Acetylation of Hydrolysed "A."

(a) *With acetic anhydride and pyridine.*—A quantitative acetylation of hydrolysed "A" was attempted by the method of PETERSON and WEST (1927). 1·004 gm. of hydrolysed "A" (m.p. 132° C.) were dissolved in 20 c.c. of a mixture of 8 c.c. of acetic anhydride and 40 c.c. of pure pyridine and incubated at 37° C. for five days in a tube fitted with a glass stopper. The contents of the tube were then washed out with 200 c.c. of chilled water and titrated with NaOH.

TABLE X.

| Weight of Hydrolysed "A." | c.c. N/1 NaOH required to Neutralise. | c.c. N/1 NaOH required to Neutralise Blank. | c.c. N/1 NaOH. Excess of Estimation over Blank. | c.c. N/1 NaOH due to Original Acidity. | c.c. N/1 NaOH equivalent to Acetic Anhydride used up. | Number of Acetyl Groups in 1 Mol. of Hydrolysed "A." |
|---------------------------|---------------------------------------|---|---|--|---|--|
| 1·004 | 70·49 | 66·08 | 4·41 | 6·12 | 1·71 | 0·58 |

The figure for the number of acetyl groups (0.58) is almost identical with those obtained in similar experiments with Substance "A" (0.55, 0.57, see p. 308), but in both cases very considerable darkening in colour occurred and the products consist of for the most part of tar. It seems unlikely, therefore, that this reaction represents a true acetylation.

(b) *Acetylation of hydrolysed "A," with acetyl chloride.*—4.2 gm. of hydrolysed "A" (m.p. 134° C.) were boiled on a water bath under reflux for three-quarters of an hour with 40 c.c. of redistilled acetyl chloride. Hydrochloric acid was evolved and the substance gradually dissolved. The excess of acetyl chloride was removed and the residue transferred by washing out with ether to a large weighed crystallising dish. After removal of the ether the viscous material was spread out in a thin layer over the dish, and was then dried *in vacuo* over strong potassium hydroxide solution and sulphuric acid. The product weighed 4.21 gm. and was partially crystalline.

It was dissolved in boiling ether and a little boiling light petroleum added. The addition of light petroleum precipitates first a quantity of Substance "A," and if sufficient is added brings down also the acetyl compound. By repeated fractionation with ether-light petroleum 2.24 gm. of Substance "A" were recovered, the melting point of the different fractions varying from 140° to 142° C. From the ether mother-liquor, 1.53 gm. of a sticky non-crystalline material were obtained after drying. This material was boiled three times with light petroleum, the petroleum poured off and evaporated. A little oily matter was thus separated and after drying for two days 1.22 gm. of acetyl derivative remained. This was dissolved in absolute alcohol and made up to 50 c.c. Of this 25 c.c. were pipetted and boiled under reflux with 20 c.c. of N/1 NaOH for 2½ hours. The hydrolysed material was acidified with H₂SO₄ and the acetic acid distilled off *in vacuo*. The combined distillates from 0.61 gm. of the acetyl derivative required 15.46 c.c. of N/10 NaOH. The theoretical value for a monoacetyl derivative is 15.72 c.c. A white precipitate which formed in the boiling flask was separated, dried and weighed (weight = 0.481 gm.). On recrystallising from ether-light petroleum, it melted at 145° C. and was evidently Substance "A." The theoretical amount of Substance "A" obtainable from 0.61 gm. of a monoacetyl derivative of hydrolysed "A" is 0.515 gm.

In a second experiment the distillate from 1.0798 gm. of the acetyl derivative required 27.45 c.c. of N/10 NaOH (theory for a monoacetyl derivative = 27.8 c.c.), and the weight of Substance "A" recovered by extraction with ether of the liquid remaining in the hydrolysis flask was 0.910 gm. (theory = 0.913 gm.). On recrystallising from ether-light petroleum and finally from water it melted at 145° C. and gave a mixed m.p. with a pure specimen of Substance "A" of 145° C.

The action of acetyl chloride on hydrolysed Substance "A" is therefore to form the mono-acetyl derivative, and also, to almost an equal extent, to dehydrate hydrolysed "A" giving Substance "A."

The acetyl derivative is an intensely sticky substance which does not crystallise on

keeping *in vacuo* for several weeks, but which on standing in the open air readily decomposes, the smell of acetic acid becoming evident and the material crystallising in the course of three days. This product, on recrystallising twice from ether and light petroleum, melted at 145° C. and gave a mixed m.p. of 145° C. with a pure sample of Substance "A." The acetyl derivative breaks down therefore on standing in air into Substance "A" and acetic acid.

Conversion of hydrolysed Substance "A" into Substance "A" by heating at 100° C.

About 0.5 gm. of hydrolysed Substance "A" was dried at 100° C. for 168 hours. At the end of this time the material had shrunk considerably and showed signs of oily droplets on the surface of the weighing bottle. The material was crystallised twice from ether-light petroleum and then dried for one week at 60° C. This recrystallised material then had the following characteristics: It melted at 145.5°–146° C. and its melting point was unchanged on mixing with Substance "A." It crystallised from water in the shining plates characteristic of Substance "A." 0.1981 gm. of it required 11.82 c.c. of N/10 sodium hydroxide corresponding to an equivalent of 167.6. Excess of alkali was now added and the alkaline mixture incubated overnight at 60° C. when a further 5.95 c.c. of N/10 acid had been produced. Thus the acidity of the material had been increased 50 per cent. by heating with dilute sodium hydroxide solution exactly as with Substance "A."

The ether-light petroleum mother-liquors left, on evaporation, a waxy material reminiscent of dehydrated "A" (p. 317), and it appears that on heating at 100° C. hydrolysed Substance "A" which has been crystallised from ether-light petroleum, three reactions take place:—

- (a) A very quick reaction, *i.e.*, loss of solvent by hydrolysed "A."
- (b) A quick reaction, *i.e.*, hydrolysed "A" \longrightarrow Substance "A."
- (c) A slow reaction, *i.e.*, Substance "A" \longrightarrow dehydrated "A."

DEHYDRATED SUBSTANCE "A."

It has been noted above that when hydrolysed Substance "A" is heated at 100° C. for a week, Substance "A" is re-formed together with a small amount of a waxy material to which the name "dehydrated Substance 'A'" is given. The quantitative relations between Substance "A," hydrolysed Substance "A," and dehydrated Substance "A" will now be dealt with, and later (p. 317) a description of the preparation and properties of dehydrated Substance "A" is given.

(a) *Effect of heating Substance "A" at 180° C.*—0.7470 gm. of air-dried Substance "A" was heated for one hour at 180° C. in a castor oil bath in a stream of dry CO₂-free nitrogen. The nitrogen, carrying products of decomposition, was passed through a calcium chloride tube to absorb any water given off and then through a baryta bubbler to absorb CO₂.

No decomposition was apparent at the melting point of Substance "A" (145° C.) but as the temperature rose bubbles of gas were given off. The temperature was maintained at 180° C. until this gas evolution ceased (one hour's heating). On cooling, the liquid in the heating tube set to a mass of fine needles. The following results were obtained :—

| | Gm. |
|---|--------|
| Weight of Substance "A" heated | 0·7470 |
| Total loss in weight of Substance "A" | 0·0452 |
| Gain in weight of calcium chloride tube | 0·0423 |
| Weight of CO_2 evolved | 0·0011 |

These results indicate that only a negligible amount of CO_2 is evolved and that the loss in weight is due to loss of water which amounts to 5·66 per cent. (calculated from the gain in weight of the calcium chloride tube). This agrees very well with the theoretical for the following equation: $\text{C}_{17}\text{H}_{28}\text{O}_6 \rightarrow \text{C}_{17}\text{H}_{26}\text{O}_5 + \text{H}_2\text{O}$ (= 5·49 per cent.).

(b) *Effect of heating hydrolysed "A" at 180° C.*—A quantity of hydrolysed Substance "A" which had been crystallised from ether-light petroleum and air-dried for 13 days was heated for one hour at 180° C., in the same apparatus and under the same conditions as in the previous experiment.

Moisture was given off as soon as the melting point was reached (133° C.) and the substance appeared to melt and then partially to re-solidify. It melted a second time at 145° C. and continued to give off gas bubbles as the temperature was raised. This gas evolution was completed some time before heating was stopped.

The following results were obtained :—

| | Gm. |
|---|--------|
| Weight of hydrolysed "A" heated | 0·6773 |
| Total loss in weight of hydrolysed "A" | 0·0942 |
| Gain in weight of calcium chloride tube | 0·0710 |
| Weight of CO_2 evolved | Nil |

Since no CO_2 at all was evolved it is evident that some substance has been given off which is neither water nor CO_2 , and amounting to 0·0942 gm. — 0·0710 gm. = 0·0232 gm. = 3·43 per cent.

This was shown to be solvent and hence the weight of solvent-free hydrolysed "A" which was used is 0·6773 gm. — 0·0232 gm. = 0·6541 gm. Hence the percentage loss of water from solvent free material is

$$\frac{0\cdot0710 \times 100}{0\cdot6541} = 10\cdot85 \text{ per cent.}$$

This agrees well with the theoretical value for the equation $\text{C}_{17}\text{H}_{30}\text{O}_7 \rightarrow 2\text{H}_2\text{O} + \text{C}_{17}\text{H}_{26}\text{O}_5$ (= 10·41 per cent.).

Perfectly definite proof was now found by the following experiment for the belief that some material, probably solvent, other than water is evolved on heating air-dried hydrolysed "A." A quantity of hydrolysed "A" which had been dried in air for eight days was heated in an apparatus similar to that used in the two above experiments, the conditions of heating, length of time, etc., being exactly the same. The gases evolved on heating were passed through two calcium chloride tubes to absorb water. The residual vapours were then passed through an ordinary combustion tube filled with copper oxide and heated in a furnace. The products of combustion were absorbed in calcium chloride and potassium hydroxide exactly as in an ordinary combustion. The following results were obtained :—

| | Gm. |
|--|--------|
| Weight of hydrolysed "A" heated.. .. | 0·8117 |
| Total loss in weight of hydrolysed "A" | 0·1127 |
| Gain in weight of calcium chloride tube | 0·0825 |
| (= 10·56 per cent. calculated on solvent free material). | |
| | Gm. |
| Therefore, weight of solvent oxidized | 0·0302 |
| Weight of water produced on combustion | 0·0341 |
| Weight of CO ₂ produced on combustion | 0·0661 |

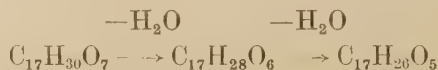
These figures correspond to 3·72 per cent. of solvent in the original air-dried hydrolysed "A," the solvent containing 12·6 per cent. hydrogen and 59·7 per cent. carbon, which figures agree reasonably well with the theoretical for diethyl ether, viz., hydrogen = 13·5 per cent. carbon = 64·9 per cent.

To sum up then, the effect of heating hydrolysed "A" or Substance "A" is that one or more of the following reactions takes place :—

(a) *Loss of solvent by hydrolysed "A."*—This change takes place, though only slowly, at room temperature, more quickly at 60° C. and very quickly at 100° C.

(b) *Hydrolysed "A" → Substance "A."*—This change takes place fairly readily at 100° C., and by careful regulation it is possible to obtain almost pure Substance "A" from hydrolysed "A" by this method. The change takes place according to the equation $C_{17}H_{30}O_7 \rightarrow C_{17}H_{28}O_6 + H_2O$

(c) *Hydrolysed "A" → Substance "A" → Dehydrated "A."*—This change takes place quickly at 180° C., though there is evidence that it also takes place very slowly at 100° C. (p. 314). The change occurs according to the following equation :—



The reverse reaction from Substance "A" to hydrolysed "A" is readily brought about by boiling with dilute alkali.

Preparation and properties of dehydrated Substance "A."

A quantity of Substance "A" was heated for an hour at 180°C . On cooling, the liquid set to a mass of fine needles which were crystallised by dissolving in a small amount of ether, adding about three volumes of light petroleum, filtering and allowing the filtrate to stand in a rapid current of air. When a sufficient degree of cooling had been obtained, the substance crystallised out, was filtered, recrystallised and air-dried. It was noted that the solubility-temperature gradient is very steep and that once the material has crystallised, rapid filtration must follow, otherwise the substance redissolves.

Dehydrated Substance "A" crystallises in fine white needles melting at 41°C .

1.9007 gm. of dehydrated Substance "A," dissolved in alcohol, required 12.27 c.c. of N/1 NaOH for neutralisation to phenolphthalein, corresponding to an equivalent of 154.9. (Theoretical for $\text{C}_{17}\text{H}_{26}\text{O}_5$, assuming this to be a dibasic acid = 155.0.)

The results of micro-combustion (SCHOELLER, Berlin) are given in Table XI.

TABLE XI.

| Weight of Dehydrated Substance "A" analysed. | Weight of CO_2 . | Weight of H_2O . | Percentage Carbon. | Percentage Hydrogen. |
|--|---------------------------|----------------------------------|--------------------|----------------------|
| Mgm. | Mgm. | Mgm. | | |
| 4.618 | 11.125 | 3.67 | 65.71 | 8.92 |
| 4.491 | 10.845 | 3.51 | 65.86 | 8.75 |
| Theoretical for $\text{C}_{17}\text{H}_{26}\text{O}_5$ | ... | ... | 65.75 | 8.45 |

Hydrolysis of Dehydrated Substance "A."

It was expected that, since dehydrated Substance "A" ($\text{C}_{17}\text{H}_{26}\text{O}_5$) arises from Substance "A" ($\text{C}_{17}\text{H}_{28}\text{O}_6$) by heating, it should be possible to regenerate Substance "A," or hydrolysed Substance "A" ($\text{C}_{17}\text{H}_{30}\text{O}_7$), by hydrolysing dehydrated Substance "A" with dilute alkali. This change does not appear to take place, however, as, on hydrolysis of dehydrated Substance "A," a new product arises which has been called Hydrolysed Dehydrated "A," and there was no evidence of the formation of either Substance "A," or Hydrolysed Substance "A."

That dehydrated "A" is hydrolysed by boiling with dilute alkali was shown quantitatively as follows:—

0.9530 gm. of dehydrated "A" was neutralised with N/2 NaOH and required 12.97 c.c. An excess of N/2 NaOH was now added, the mixture boiled and titrated back.

Acid equivalent to a further 5.68 c.c. of N/2 NaOH was formed, so that the equivalent of the hydrolysis product is 108.1.

In a repeat experiment on similar lines an equivalent of 111.0 was obtained.

The values agree with the theoretical value for a compound having the same empirical formula as Substance "A" ($C_{17}H_{28}O_6$) but possessing *three* carboxyl groups (theoretical = 109.3).

Isolation and properties of hydrolysed dehydrated "A."

Considerable difficulty was experienced in isolating the substance obtained by boiling neutralised dehydrated "A" with excess of sodium hydroxide. The substance very readily decomposes in presence of acid. Thus, acidification, with *normal* hydrochloric or sulphuric acid, of the alkaline solution after hydrolysis and shaking out with ether, gave a product consisting almost exclusively of dehydrated "A." When, however, the diluted hydrolysed solution was acidified with N/10 HCl in presence of ether, the acid being added in small quantities with continuous shaking, it was found that on evaporating the ethereal solution in a current of air to small bulk and adding an equal volume of light petroleum, a good yield of an acid was precipitated, having a melting point of 87° C.

The action of heat on hydrolysed dehydrated "A."

0.4789 gm. of hydrolysed dehydrated "A" (m.p. 87° C.) was heated for one hour at a temperature rising from 100° to 160° C. in a castor oil bath in a stream of dry CO₂-free nitrogen. The substance decomposed almost immediately after melting, and the water formed was carried in the stream of nitrogen through weighed calcium chloride tubes. On cooling, the liquid in the heating tube set to a mass of fine needles. The residual material on crystallising from ether-light petroleum melted at 41° C. and gave an equivalent of 148.9, and was therefore dehydrated Substance "A." The following results were obtained:—

| | Gm. |
|--|--------|
| Weight of hydrolysed dehydrated "A" | 0.4789 |
| Total loss in weight | 0.0379 |
| Gain in weight of the calcium chloride tubes | 0.0264 |

The gain in weight of the calcium chloride tubes corresponds to a loss of water amounting to 5.51 per cent., which agrees very closely with the theoretical for the following equation (5.49 per cent.): $C_{17}H_{28}O_6 \rightarrow C_{17}H_{26}O_5 + H_2O$. It would appear, therefore, that hydrolysed dehydrated "A" is a substance having the same empirical formula as Substance "A," but containing three carboxyl groups and on heating above 100° C. it reverts by loss of 1 mol. of water to dehydrated "A."

The presence of solvent of crystallisation, as indicated by the difference between the *total* loss in weight on heating (0.0379 gm.) and the gain in weight of the CaCl₂ tubes (0.0264 gm.), together with the extreme instability of hydrolysed dehydrated "A"

even at room temperature, have rendered it impossible to obtain satisfactory combustion results. The instability of the material is indicated by the fact that, while a freshly prepared sample has a melting point of 87°C ., this had fallen to 82°C . after standing for three days at room temperature and to 42° – 43°C . after standing for three weeks at the same temperature.

ALKYLATION PRODUCTS.

It was hoped by studying the alkylation products of Substance "A" and hydrolysed Substance "A" to obtain confirmation of the observation already made (see p. 313) that hydrolysed "A" contains one hydroxyl group. The alkylation products were, however, disappointing, and it was found impossible to alkylate the hydroxyl group by any method tried, although the three carboxyl groups were relatively easily esterified. Further, all the products obtained were oily liquids which refused to crystallise and were difficult to purify.

1. *Methylation of Substance "A" with diazomethane.*

One gram of pure Substance "A" was dissolved in dry ether and an excess of an ethereal solution of diazomethane was added. An immediate reaction took place. After standing for one hour the excess of diazomethane was removed by shaking with a little water. The ethereal solution was separated, evaporated and dried to constant weight over H_2SO_4 in a vacuum desiccator. 1.02 gm. of a colourless oil were obtained, which did not crystallise on standing for several weeks.

ZEISEL estimations on this material gave the following results:—0.1353 gm. and 0.1204 gm. respectively gave 0.1770 gm. and 0.1574 gm. of silver iodide, corresponding to 17.26 per cent. and 17.25 per cent. OCH_3 (theoretical for $\text{C}_{17}\text{H}_{26}\text{O}_4$ $(\text{OCH}_3)_2 = 17.42$ per cent.).

2. *Methylation of Hydrolysed Substance "A" with dimethyl sulphate.*

A quantity of hydrolysed "A" was treated with a large excess of methyl sulphate, sodium hydroxide added and the mixture boiled for about an hour, the reaction being kept alkaline by the addition of more sodium hydroxide. The mixture was cooled, filtered and acidified, giving rise to a white gelatinous precipitate which was extracted with ether and fractionally crystallised from ether and light petroleum. It was shown to consist of unchanged hydrolysed Substance "A," and there was no indication of the formation of any methoxyl compound.

3. *Ethylation of hydrolysed Substance "A" with diazoethane.*

2 gm. of pure hydrolysed Substance "A" were dissolved in dry ether and a considerable excess of an ethereal solution of diazoethane added. An immediate reaction

took place and the mixture was allowed to stand overnight. The excess of diazoethane was then removed in the ether vapour by evaporation and the resultant pale yellow oil was dried over sulphuric acid. ZEISEL estimations on this material gave the following results:—0.1356 gm. and 0.1284 gm. respectively gave 0.2197 gm. and 0.2073 gm. of silver iodide, corresponding to 31.08 per cent. and 30.96 per cent. OC_2H_5 (theoretical for $\text{C}_{17}\text{H}_{28}\text{O}_5$ (OC_2H_5)₂ = 22.39 per cent. and $\text{C}_{17}\text{H}_{27}\text{O}_4$ (OC_2H_5)₃ = 31.39 per cent.). The ethylation product is thus either a triethyl ester or a monoethyl ether of a diethyl ester.

It was shown that the product does not contain an ethoxyl group, by hydrolysing a quantity of it with boiling alcoholic sodium hydroxide. The regenerated acid melted at 132° C., gave an equivalent of 121.2, and was unchanged hydrolysed Substance "A." Hence, this ethylation product is the triethyl ester of hydrolysed Substance "A." This conclusion is confirmed by the fact that this product is insoluble in sodium hydroxide.

4. *Attempted methylation of the triethyl ester of hydrolysed "A" with methyl iodide and silver oxide.*

An attempt was made to methylate the triethyl ester of hydrolysed Substance "A" by the method of PURDIE and IRVINE (1903). 1 gm. of the triethyl ester was treated with 2.18 gm. of methyl iodide and 1.79 gm. of silver oxide in 7 gm. of methyl alcohol. After refluxing for two hours the ester was recovered. It still gave a ZEISEL value of 31.18 per cent. OC_2H_5 , and on hydrolysis by heating with alcoholic sodium hydroxide, unchanged hydrolysed "A" was obtained. Methylation, therefore, did not appear to have taken place. It is interesting to note that a similar difficulty was experienced by ANSCHÜTZ (1903), in an attempt to methylate the hydroxyl group of citric acid.

Amides of Substance "A" and Hydrolysed Substance "A."

(a) *Amides from distilled methylated Substance "A."*—1 gm. of the twice distilled dimethyl compound of Substance "A" was treated with about ten volumes of aqueous ammonia solution saturated at 0° C., shaken and kept in a stoppered tube overnight at 0° C. In the morning the mixture appeared as a thin, gelatinous, soapy mass which was dried completely over sulphuric acid and then crystallised from boiling absolute alcohol. On cooling, a small amount of material (fraction 1) separated, consisting of very fine microscopic needles. The mother-liquors from fraction 1 were evaporated to dryness and crystallised from a mixture of ether and light petroleum, giving rise to a crystalline material (fraction 2). The mother-liquors from fraction 2 were freed from solvent, re-treated with 10 volumes of ammonia, and the fractionation carried through again, as described above. By this means further quantities of fractions 1 and 2 were obtained.

Fraction 1.—0.2 gm. of crude fraction 1 was obtained. This was recrystallised from absolute alcohol and was finally obtained as a white powder crystallising in fine white needles and having a melting point of 178.5° C. It gave the following results on analysis (SCHOELLER, Berlin):—

5.188 mg. gave 11.450 mg. CO_2 and 4.40 mg. H_2O . C = 60.20 per cent., H = 9.50 per cent. (Duplicate C = 60.51 per cent., H = 9.66 per cent.)

3.047 mg. gave 2.12 mg. Ag I. OCH_3 = 9.19 per cent. (Duplicate 8.90 per cent.)

3.065 mg. gave 0.211 c.c. nitrogen at 24.5° C. and 762 m.m. Nitrogen = 7.92 per cent. (Duplicate 7.78 per cent.)

Calculated for $\text{C}_{18}\text{H}_{34}\text{O}_5\text{N}_2 = \text{C}_{17}\text{H}_{31}\text{O}_4\text{N}_2 (\text{OCH}_3)$. C = 60.34 per cent., H = 9.56 per cent., N = 7.82 per cent., OCH_3 = 8.66 per cent.

Fraction 2.—0.60 gm. of fraction 2 was obtained, which on recrystallisation from ether-light petroleum separated in beautiful white prisms melting at 78.5° C. which gave the following results on analysis (SCHOELLER, Berlin):—

4.382 mg. gave 9.850 mg. CO_2 and 3.65 mg. H_2O . C = 61.31 per cent., H = 9.32 per cent. (Duplicate C = 61.31 per cent., H = 9.45 per cent.)

3.041 mg. gave 4.24 mg. AgI. OCH_3 = 18.42 per cent. (Duplicate 18.08 per cent.)

2.925 mg. gave 0.098 c.c. nitrogen at 23.5° C. and 762 m.m. Nitrogen = 3.87 per cent. (Duplicate 3.95 per cent.)

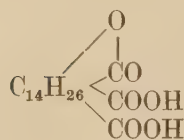
Calculated for $\text{C}_{19}\text{H}_{35}\text{O}_6\text{N} = \text{C}_{17}\text{H}_{29}\text{O}_4\text{N} (\text{OCH}_3)_2$. C = 61.08 per cent., H = 9.45 per cent., N = 3.75 per cent., OCH_3 = 16.63 per cent.

(b) *Amides from undistilled methylated Substance "A."*—5 gm. of crude, undistilled methylated Substance "A" were treated exactly as described in paragraph (a) for the distilled material. The yields of amides obtained were in marked contrast to those obtained with the distilled methyl compound since, while 3.1 gm. of fraction 1 were obtained, it was found impossible to isolate any fraction 2. The 3.1 gm. of fraction 1, on recrystallisation from absolute alcohol melted at 178.5° C. and admixture with a little of fraction 1 from the distilled methyl compound produced no lowering of the melting point. The two fractions may therefore be taken to be identical.

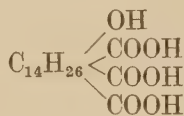
(c) *Amides from triethyl ester of hydrolysed Substance "A."*—On treating the triethyl ester of hydrolysed Substance "A" with cold saturated aqueous ammonia the greater part of the triethyl ester was recovered unchanged even after lengthy treatment. A small amount of the diamide was, however, obtained having a melting point of 178.5° C. and a mixed melting point of 178° C. with fraction 1.

From the analytical results it appears that fraction 1 (m.p. 178.5° C.) is the diamide of the monomethyl ester of hydrolysed Substance "A," while fraction 2 (m.p. 78.5° C.)

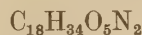
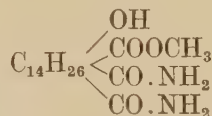
is the monoamide of the dimethyl ester of hydrolysed Substance "A" Their relations to Substance "A" and to hydrolysed Substance "A" are indicated as follows :—



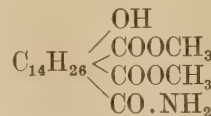
Substance A.



Hydrolysed
Substance A.



Fraction 1.
M.Pt. 178.5° C.



Fraction 2.
M.Pt. 78.5° C.

Fusion of Substance "A" with Potassium hydroxide. Isolation of Lauric Acid (C₁₂H₂₄O₂).

1 gm. of Substance "A," 5 gm. of potassium hydroxide and 3 c.c. of water were warmed until the material was completely dissolved, and then heated in a metal bath at 310° C. for one hour. After cooling, the melt was dissolved in about 15 c.c. of water and acidified with 110 c.c. of N/1 H₂SO₄. Carbon dioxide was liberated and a substance precipitated, which on drying weighed 0.5 gm. It was dissolved in ether, filtered, the ethereal solution treated with a little animal charcoal to remove a trace of yellow oil, and the clear filtrate evaporated. The residue melted at 41° C. and gave an equivalent by titration of 196.5 (0.1031 gm. required 5.42 c.c. of N/10 NaOH). It was crystallised to constant melting point from light petroleum and air-dried. This product then melted at 43.6° C. and gave an equivalent of 199.2 (0.1998 gm. required 10.03 c.c. of N/10 NaOH). Lauric acid, C₁₂H₂₄O₂, melts at 43.6° C., and has an equivalent of 200. The combustion figures are as follows (SCHOELLER, Berlin) :—

TABLE XII.

| Weight of Fusion Product | Weight of CO ₂ . | Weight of H ₂ O. | Percentage Carbon. | Percentage Hydrogen. |
|--|-----------------------------|-----------------------------|-----------------------|-------------------------|
| Mgm. | Mgm. | Mgm. | | |
| 5.070 | 13.430 | 5.50 | 72.26 | 12.14 |
| 3.708 | 9.770 | 4.02 | 71.88 | 12.13 |
| Theoretical for C ₁₂ H ₂₄ O ₂ | ... | ... | 71.96 | 12.09 |

The mixed melting point of the product with a pure sample of lauric acid kindly supplied by Professor R. ROBINSON, F.R.S., was also 43.6° C., and lauric acid is therefore one product of fusion.

The solution remaining after filtering off the lauric acid was extracted with ether in a continuous extractor for four days and gave 0.18, 0.16, 0.06 and 0.03 = 0.43 gm. of dried material. This material was shown to consist of a mixture of oxalic and succinic

acids. It was dissolved in water and the oxalic acid removed as calcium oxalate. The oxalic acid was regenerated from the calcium oxalate and sublimed in a high vacuum. The sublimate melted at 187°C . and had an equivalent of 45.5 (0.1021 gm. required 22.44 c.c. of N/10 NaOH). The oxalate-free solution was now treated with barium chloride, heated, the precipitated barium succinate filtered off, acidified, and the succinic acid extracted with ether and sublimed. It had a melting point of 184°C . and an equivalent of 60.1 (0.0119 gm. required 1.98 c.c. of N/10 NaOH).

The products obtained by fusion of 1 gm. of Substance "A" with potassium hydroxide are therefore 0.5 gm. of a material consisting almost entirely of lauric acid, a considerable amount of carbon dioxide, and 0.43 gm. of a mixture of succinic and oxalic acids. The oxalic acid probably arises from succinic acid under the conditions of fusion while the carbon dioxide is probably produced from formic acid, since sodium formate is known to break down readily under these conditions. It seems probable, therefore, that the initial products obtained by fusion from 1 molecule of Substance "A" are 1 molecule each of lauric, formic and succinic acids.

Oxidation of Substance "A" with acid permanganate.

2 gm. of Substance "A" were dissolved in 75 c.c. of N/1 H_2SO_4 and heated under reflux in a boiling water bath. 50 c.c. of a solution containing 0.77 gm. of KMnO_4 were now added, a few drops at a time. The permanganate solution was decolorised almost immediately, providing the addition was not too rapid. A considerable amount of oily material was gradually precipitated as the reaction proceeded. After all the permanganate had been added, the reaction was stopped and the flask cooled, when the oil readily solidified and was filtered off, dried and weighed. Weight = 1.54 gm. This material softened at 74°C . and completely melted at 103°C . By dissolving in ether and adding light petroleum 1.1 gm. of the material were precipitated as a white solid having a m.p. of 140°C ., which on recrystallisation from water had a m.p. of 145°C . and was unchanged Substance "A." The material remaining in solution was again fractionated from ether-light petroleum and a further 0.1 gm. of impure Substance "A" separated. The ether-light petroleum filtrate on evaporating in a current of air now gave a residue having a weight of 0.29 gm., a m.p. of 87°C . and an equivalent of 229.8 (0.0878 gm. required 3.82 c.c. of N/10 NaOH).

In a repeat experiment 2.14 gm. of Substance "A" gave rise to 0.2 gm. of the oxidation product having a m.p. of 87°C . This was recrystallised to constant melting point from boiling light petroleum from which it separates very readily on cooling in glistening plates having a m.p. of 92.6°C . and an equivalent of 250.7 (0.1070 gm. required 4.27 c.c. of N/10 NaOH). On combustion it gave results as shown in Table XIII (SCHOELLER, Berlin).

Both the equivalent and the results of combustion correspond very closely with those required for ketopentadecic acid $\text{C}_{15}\text{H}_{28}\text{O}_2$ (equivalent 256).

TABLE XIII.

| Weight of Substance Analysed. | Weight of CO ₂ . | Weight of H ₂ O. | Percentage Carbon. | Percentage Hydrogen. |
|---|-----------------------------|-----------------------------|--------------------|----------------------|
| Mgm. 4.758 | Mgm. 12.235 | Mgm. 4.70 | 70.14 | 11.05 |
| 4.243 | 10.920 | 4.21 | 70.19 | 11.10 |
| Theoretical for C ₁₅ H ₂₈ O ₃ | | | 70.26 | 11.01 |

In view of the small yields of oxidation product obtained a further experiment was carried out in which considerably larger amounts of permanganate were used. In this case, however, none of the product C₁₅H₂₈O₃ was obtained, the main oxidation product proving to be succinic acid.

Oxidation of hydrolysed "A" with alkaline permanganate.

The amount of ketopentadecic acid obtained by acid oxidation was never large (0.1–0.2 gm. from 2 gm. of Substance "A") and other methods of oxidation were tried in order to obtain a better yield so that complete identification would be more readily possible.

Oxidation by alkaline permanganate, however, did not yield more than a trace of the keto-acid. In one experiment 2.5 gm. of Substance "A" were boiled with 50 c.c. of N/1 NaOH for one hour in order to open the lactone ring, diluted to 1,500 c.c. and 1,500 c.c. of permanganate (containing 15 gm. of KMnO₄) and 3 gm. of sodium hydroxide were added. The solution was heated in a boiling water bath for one hour, cooled, sufficient sulphur dioxide passed just to clear the liquid and 20 c.c. of N/1 H₂SO₄ added. A white precipitate was obtained which on filtering off and drying weighed 1.2 gm. and consisted almost entirely of hydrolysed "A" together with a trace of the keto-acid which was separated by its solubility in boiling light petroleum. From the aqueous solution, after neutralising, evaporating *in vacuo* to a small bulk, acidifying and extracting in a continuous extractor with ether, there was obtained 1.18 gm. of material consisting of a yellow oil, together with a small amount of volatile acid reminiscent of butyric and caproic acids, and a larger amount of succinic acid which, after subliming twice, melted at 184° C. and gave an equivalent of 59.9 (0.01706 gm. required 2.85 c.c. of N/10 NaOH).

It appears, therefore, that oxidation with alkaline permanganate probably gives rise to the keto-acid, but that this is further oxidized yielding successively the series of fatty acids containing an even number of carbon atoms from lauric acid downwards, together with succinic acid.

Oxidation of hydrolysed "A" with permanganate in acetone solution. Preparation of γ -ketopentadecic acid.

4 gm. of hydrolysed "A," m.p. 134°C ., were dissolved in 60 c.c. of acetone and 16 c.c. of water added. The solution was then warmed in a water bath until the acetone began to boil and 0.732 gm. of powdered potassium permanganate added, a little at a time. The permanganate immediately disappeared and carbon dioxide was evolved. When all the permanganate had been added, the acetone was evaporated, the residue cooled, 50 c.c. of N/10 H_2SO_4 added and sufficient sulphur dioxide passed, just to clear the solution. The product was then extracted with ether, the ether removed and the residue dried and weighed (Wt. = 2.8 gm.). This was then digested with a large volume of boiling light petroleum in which it almost completely dissolved. On filtering the hot solution the keto-acid immediately crystallised in plates melting at 88°C . It was then dissolved in ether, treated with a little animal charcoal to remove a small amount of a yellow oil and after filtering and removing the ether, was crystallised twice from boiling petroleum. The product then melted at 92.6°C . Its melting point was unchanged on admixture with the keto-acid prepared by oxidation with permanganate in sulphuric acid solution. It also had the same equivalent, 0.1287 gm. requiring 5.10 c.c. of N/10 NaOH corresponding to an equivalent of 252.3 (theoretical for $\text{C}_{15}\text{H}_{28}\text{O}_3$ = 256). It will be noticed that the yield of crude keto-acid obtained by this method from hydrolysed "A" is over 90 per cent.

Reduction of γ -ketopentadecic to n-pentadecic acid.

0.5 gm. of γ -ketopentadecic acid, prepared from hydrolysed "A" as described above, was reduced by CLEMMENSEN'S method (1914). 50 gm. of zinc were immersed for one hour in 300 c.c. of 5 per cent. mercuric chloride solution. The aqueous layer was poured off and the Zn-Hg couple used without further washing. The Zn-Hg couple and the ketopentadecic acid were now boiled for one hour with dilute hydrochloric acid, further quantities of acid being added from time to time. After cooling, the product was extracted with ether, the ether removed and the residue dried (weight = 0.48 gm.). The dry residue dissolved almost completely in cold light petroleum and on blowing a current of air over the solution, n-pentadecic acid crystallised out. It was recrystallised from light petroleum and had the following properties: it melted at 53°C . and its melting point was not altered by admixture with a pure sample of n-pentadecic acid prepared from material received through the courtesy of Prof. A. C. CHIBNALL. The reduction product had an equivalent of 243.4, 0.1478 gm. requiring 6.06 c.c. of N/10 NaOH—while the synthetic product had an equivalent of 242.6, 0.2382 gm. requiring 9.82 c.c. of N/10 NaOH—and the theoretical for $\text{C}_{15}\text{H}_{30}\text{O}_2$ is 242.2. The reduction product gave the following figures on combustion (Table XIV, SCHOELLER, Berlin):—

TABLE XIV.

| Weight of Reduction Product Analysed. | Weight of CO ₂ . | Weight of H ₂ O. | Percentage Carbon. | Percentage Hydrogen. |
|--|-----------------------------|-----------------------------|-----------------------|-------------------------|
| Mgm. | Mgm. | Mgm. | | |
| 2.948 | 8.030 | 3.37 | 74.29 | 12.78 |
| 4.554 | 12.410 | 5.09 | 74.35 | 12.51 |
| Theoretical for C ₁₅ H ₃₀ O ₂ | | | 74.35 | 12.49 |

*Preparation of γ -ketopentadecoic acid from synthetic *n*-pentadecoic acid.*

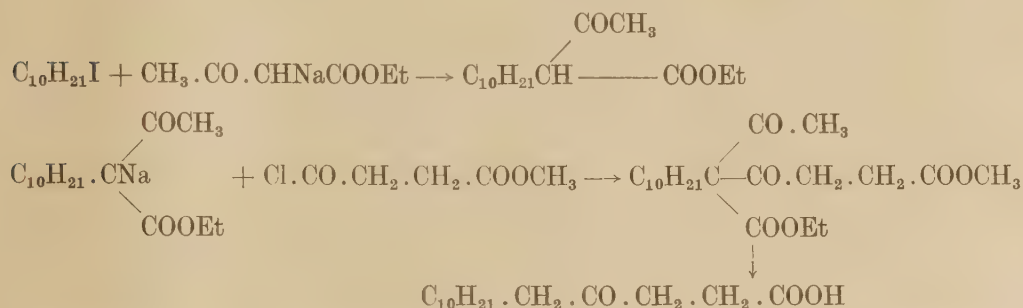
The keto-acid was prepared by the method of CLUTTERBUCK and RAPER (1925) by oxidation of the ammonium salt of *n*-pentadecoic acid with hydrogen peroxide. The *n*-pentadecoic acid was purchased from Messrs. Eastman Kodak, and purified by repeated crystallisation from light petroleum.

5 gm. of recrystallised *n*-pentadecoic acid, 3 c.c. of 0.88 ammonium hydroxide and 250 c.c. of water were heated under reflux in a large flask in the water bath. When the temperature had been raised to 60° C. and the acid completely converted to soap, 10 c.c. of "perhydrol" were added and the temperature raised gradually to 90° C. After the reaction had ceased, two further amounts of 10 c.c. of peroxide were added. The whole reaction lasted about four hours. The mixture was cooled, acidified with phosphoric acid, the products filtered off, the acids converted into the soaps with a little alcoholic potassium hydroxide and the ketone extracted with light petroleum. The residue was acidified and the acids were filtered off and crystallised twice from methyl alcohol, the liquid being cooled by blowing a current of air over the alcoholic solution. In this way 75 per cent. of the pentadecoic acid was recovered unchanged. The acid obtained in the mother liquors of three such experiments was then crystallised from light petroleum, and gave an acid which, after several recrystallisations from boiling petroleum, melted at 92.6° C. and gave a molecular weight by titration of 254 (0.1250 gm. required 4.92 c.c. of N/10 NaOH). The m.p. was not depressed by mixing with the γ -ketopentadecoic acid obtained from Substance "A" by oxidation with permanganate in acetone solution.

Synthesis of γ -Ketopentadecoic Acid.

The starting point for the synthesis was *n*-octyl iodide, which was first condensed with ethyl malonate, the ethyl octylmalonate hydrolysed and the free acid distilled to remove CO₂ thus forming *n*-decoic acid. This acid was then esterified and the ester reduced to decyl alcohol by the method of BOUVAULT and BLANC (1903) and this on treatment with hydrogen iodide (KRAFFT, 1886) gave decyl iodide. The synthesis

was then carried out by a similar method to that used by ROBINSON and ROBINSON (1925) for the synthesis of other keto-acids. Decyl iodide was condensed with sodio-acetoacetate to give ethyl α -acetyl-*n*-dodecoate which was treated with sodium and condensed with γ -carbomethoxypropionyl chloride. The product after alkaline and then acid hydrolysis gave finally γ -ketopentadecoic acid.



Ethyl n-octylmalonate.—2.3 gm. of sodium were dissolved in 25 gm. of alcohol and 16 gm. of ethyl malonate added and allowed to stand; 31 gm. of octyl iodide were then added and the mixture heated on the water bath for two hours. The alcohol was removed, the flask cooled and water added to dissolve the separated iodide. The ester was extracted with ether, the ethereal solution dried over CaCl_2 and fractionated *in vacuo*. Yield 18.5 gm. of an oil, b.p. $169^\circ\text{C}/17\text{ mm}$.

n-Octylmalonic acid.—The ester was hydrolysed by boiling for two hours with alcoholic potassium hydroxide and after removing the alcohol and cooling, the acid was precipitated with hydrochloric acid, filtered off and dried on a porous plate. The crude acid weighed 15 gm. (m.p. 108°C . decomp. at 140°C .).

n-Decoic acid.—The octylmalonic acid was then distilled *in vacuo* and gave 9 gm. of solid decoic acid m.p. 31°C .

Ethyl n-decoate.—13 gm. of *n*-decoic acid were esterified by the method of FISCHER and SPEIER (1895) using 90 c.c. of absolute alcohol and 3 gm. of dry gaseous hydrochloric acid. The alcohol was distilled off and the residue poured into water. The ester was extracted with ether, the ethereal solution washed and dried over CaCl_2 and distilled. The ester boiled at $131^\circ\text{C}/17\text{ mm}$. Yield = 11 g.

Decyl alcohol.—22 gm. of ethyl decoate were dissolved in 80 gm. of absolute alcohol (redistilled over a little sodium) and the solution dropped slowly by way of a condenser on to 16 gm. of sodium. The sodium melted and was shaken whilst the alcoholic solution was being added. After reduction was completed, a little more alcohol was added and the flask was heated in a boiling CaCl_2 bath to remove any small pieces of sodium, cooled, water added and the ethyl alcohol distilled off. The residue was then acidified and extracted with ether. The ethereal solution was then dried and distilled, the decyl alcohol being collected at $127^\circ\text{C}/16\text{ mm}$., and the decoic acid, resulting from hydrolysis of the ester, at $170^\circ\text{C}/16\text{ mm}$. The yield of distilled alcohol was 10 gm.

Decyl iodide.—The alcohol was saturated with gaseous hydrogen iodide, heated on the water bath, cooled and again saturated, allowed to stand overnight, again saturated and allowed to stand. The iodide was then decolorised with sodium carbonate and extracted with ether, the ethereal solution dried over CaCl_2 and distilled. The decyl iodide boiled at $132^\circ \text{C./15 mm.}$

Ethyl α -acetyl-n-dodecoate.—0.93 gm. of sodium was dissolved in 12 c.c. of absolute alcohol and 7.9 gm. of ethyl acetoacetate and subsequently 10.7 gm. of decyl iodide were added. The mixture was heated on the water bath under reflux for three hours. Sodium iodide separated. The alcohol was removed in a stream of air, the residue cooled, water added to dissolve the iodide and the ester extracted with ether. The ethereal solution was dehydrated over CaCl_2 and distilled. The dodecoate boiled at $170^\circ \text{C./16 mm.}$

γ -Carbomethoxypropionyl chloride.—This was prepared by the method of CLUTTERBUCK and RAPER (1925) by converting succinic anhydride into the monomethyl ester, treating with thionyl chloride and fractionating; the methyl ester chloride boiled at $93^\circ \text{C./18 mm.}$

γ -Ketopentadecoic acid.—A solution of γ -carbomethoxypropionyl chloride (4 gm.) in ether (20 c.c.) was added to the sodio derivative from ethyl α -acetyl-n-dodecoate (7 gm.) and sodium (0.6 gm.) in ether (220 c.c.). The mixture was cooled in ice water, kept overnight and boiled under reflux for one and a half hours. The washed and isolated product was hydrolysed by boiling for six hours with 5 per cent. KOH (200 c.c.), then for 30 hours with 5 per cent. H_2SO_4 (160 c.c.) and finally for three hours with 5 per cent. KOH . The solution was then acidified and extracted with ether. The ethereal solution was reduced to a small volume and the keto-acid precipitated with light petroleum. It was finally recrystallised four times from boiling light petroleum and then melted at 92.6°C. and did not depress the m.p. of the keto-acid obtained by oxidation of hydrolysed "A" with permanganate. 0.1029 gm. required 4.05 c.c. of N/10 NaOH for neutralisation, corresponding to an equivalent of 254 (theoretical for $\text{C}_{15}\text{H}_{28}\text{O}_3 = 256$).

The substance gave the following results on micro-combustion (SCHOELLER, Berlin):—

TABLE XV.

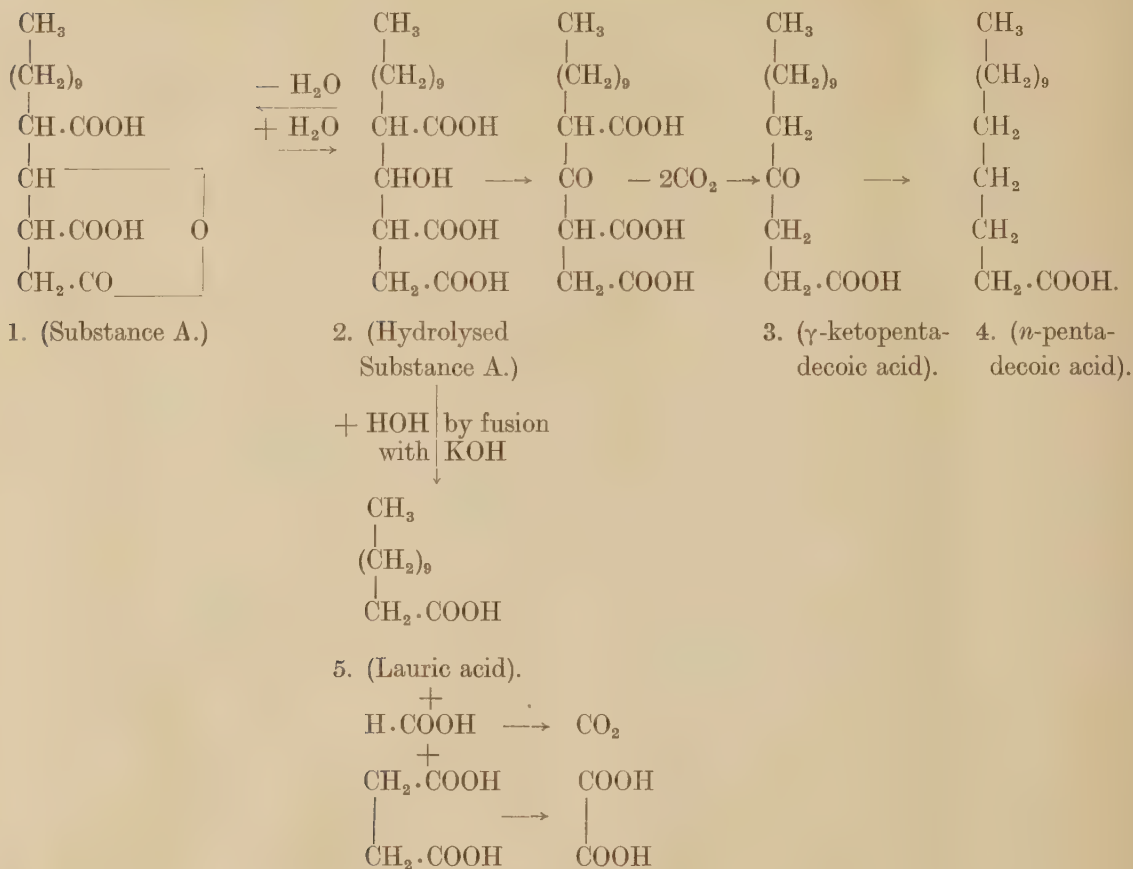
| Weight of Substance Analysed. | Weight of CO_2 . | Weight of H_2O . | Percentage Carbon. | Percentage Hydrogen. |
|--|---------------------------|----------------------------------|--------------------|----------------------|
| Mgm. | Mgm. | Mgm. | | |
| 4.737 | 12.140 | 4.63 | 69.91 | 10.94 |
| 4.991 | 12.820 | 4.90 | 70.03 | 10.98 |
| Theoretical for $\text{C}_{15}\text{H}_{28}\text{O}_3$ | ... | ... | 70.26 | 11.01 |

Discussion of Results.

The following results are of importance in arriving at a constitutional formula for Substance "A":—

1. Substance "A," $C_{17}H_{28}O_6$, titrates as a dibasic acid, contains two COOH groups (p. 305), and an asymmetric carbon atom (p. 306).
2. On hydrolysis with dilute sodium hydroxide, Substance "A," $C_{17}H_{28}O_6$, gives rise to hydrolysed Substance "A," $C_{17}H_{30}O_7$, which titrates as a tribasic acid containing three carboxyl groups (p. 310). The seventh oxygen atom is present as a hydroxyl group since hydrolysed Substance "A" gives a mono-acetyl derivative (p. 313). The ease with which hydrolysed Substance "A" reverts to Substance "A" by the loss of a molecule of water on heating (p. 314), suggests that hydrolysed Substance "A" is a γ -hydroxy-tricarboxylic acid, and that Substance "A" is the lactone of this acid.
3. Substance "A" on methylation with diazomethane gives a lactone dimethyl ester (p. 319) while hydrolysed Substance "A" on ethylation with diazoethane gives a hydroxy-triethyl ester (p. 319).
4. On fusion with potassium hydroxide, hydrolysed Substance "A" gives lauric acid, $C_{12}H_{24}O_2$, succinic acid, $C_4H_6O_4$, and carbon dioxide (p. 322).
5. Hydrolysed Substance "A" is oxidized by permanganate, in acetone solution, almost quantitatively to a ketopentadecic acid (p. 325), which on reduction gives an almost quantitative yield of *n*-pentadecic acid (p. 325).
6. The ketopentadecic acid arising as an oxidation product of hydrolysed Substance "A," was shown by synthesis to be the γ -keto-acid (p. 326), thus indicating that the hydroxyl group in hydrolysed Substance "A" is in the γ -position to the terminal carboxyl group.

These facts prove conclusively that Substance "A" is the lactone of γ -hydroxy- $\beta\delta$ -dicarboxypentadecic acid of the formula (1) and hydrolysed Substance "A" is thus γ -hydroxy- $\beta\delta$ -dicarboxypentadecic acid, formula (2). The breakdown of Substance "A" on oxidation to γ -ketopentadecic acid, formula (3) and to *n*-pentadecic acid formula (4), is shown in the following scheme in which is also given the relation of Substance "A" to its fusion products, lauric acid, formula (5), succinic acid and CO_2 .



Summary.

P. spiculisporum LEHMAN, when grown on CZAPEK-DOX glucose solution, gives rise to a new metabolic product. This substance, which is a new polybasic fatty acid, $\text{C}_{17}\text{H}_{28}\text{O}_6$, is the lactone of γ -hydroxy- $\beta\delta$ -dicarboxypentadecic acid. A description is given of its preparation, properties, derivatives and breakdown products. Other metabolic products of *P. spiculisporum* LEHMAN are succinic acid and γ -ketopentadecic acid.

*Studies in the Biochemistry of Micro-organisms.*PART XVII.—*The Products of Glucose Metabolism formed by various species of Fungi*
(*Helminthosporium*, *Clasterosporium*, etc.).

By JOHN HOWARD BIRKINSHAW and HAROLD RAISTRICK.

In the following paper an account is given of the investigation of the metabolic products formed from glucose by various species of fungi belonging to different genera. These species were chosen for investigation since the carbon balance sheets prepared for them, when grown on CZAPEK-DOX glucose solution, indicated that each of them gives rise to considerable quantities of some metabolic product. The isolation, identification and approximate estimation of these products are described in detail.

The species of fungi chosen for examination were the following :—

- (a) *Helminthosporium geniculatum* TRACY et EARLE. Catalogue No. Ag. 93. This species was purchased in 1925 from the Centraalbureau voor Schimmelcultures at Baarn. For carbon balance sheet, see Part VI, Table VIII, p. 111.
- (b) *Clasterosporium* species, Catalogue No. Ag. 64. This species was isolated at Ardeer in 1924 from decaying cotton pulp, and its diagnosis was confirmed by Mr. F. T. BROOKS, of the School of Botany, Cambridge. For carbon balance sheet, see Part VI, Table XII, p. 118.
- (c) *Aspergillus Wentii* WEHMER. Catalogue No. Ac. 81. This species was received from Miss CHURCH, of the United States Bureau of Agriculture, at Washington, and bears the THOM and CHURCH Catalogue No. 4202·16C. For carbon balance sheet, see Part III, Table XI, p. 45.
- (d) *Fumago vagans* PERS. Catalogue No. Ag. 92. This species was purchased from Baarn in 1925. For carbon balance sheet, see Part VI, Table XI, p. 116.
- (e) *Penicillium* species. Catalogue No. Ad. 11. This species was isolated at Ardeer in 1922 from mouldy tobacco and was diagnosed by Dr. CHARLES THOM as “Ad. 11—one of the *P. chrysogenum* lot.” For carbon balance sheet, see Part IV, Table V, p. 63.

The method adopted for the preparation of sufficient material for investigation with each of the above species was to make use of the combined sterilizer-incubator described in Part VII. A quantity of the normal CZAPEK-DOX glucose medium having the composition given in Part I, p. 7, was prepared, and the moulds were grown in the sterilizer-incubator in the usual way.

(a) *Helminthosporium geniculatum*.

Thirty litres of medium, distributed between six trays, were used in this experiment which commenced on 5th November, 1928.

Aeration was commenced on 13th November, 1928, 0.2 cu. ft. of air per tray per day being given for the first six days. From 20th November, 1928, to 9th January, 1929, 0.3 cu. ft. of air per tray per day was passed, and from 10th January, 1929, to the completion of the experiment on 31st January, 1929, 0.2 cu. ft. of air per tray per day was passed. Aeration was restricted throughout to six days per week. The incubation period thus lasted 87 days.

The six trays when taken off on 31st January, 1929, showed the results given in Table I.

TABLE I.

| Number of Tray. | Percentage Glucose by Polarimeter. | Percentage Glucose by WOOD-OST Method. | Titration in c.c. N/1 NaOH per 250 c.c. Medium. |
|-----------------|---------------------------------------|--|---|
| 1 | 1.09 | 0.92 | 0.75 |
| 2 | 0.56 | 0.41 | 0.30 |
| 3 | 0.86 | 0.73 | 0.20 |
| 4 | 0.80 | 0.68 | 1.15 |
| 5 | 1.59 | 1.38 | 0.70 |
| 6 | 0.30 | 0.24 | 2.25 |

All the six trays were found to be free from contamination with the exception of Tray 5, which was found to be slightly contaminated, and was therefore discarded. The contents of the other five trays were filtered from the mycelium, and the mycelium (A), and filtrate (B), dealt with separately.

A. *Treatment of mycelium*.—The mycelium was thoroughly extracted with boiling water, the extract evaporated to small bulk, *in vacuo* and precipitated with 2 vols. of 96 per cent. alcohol. A small amount of a substance resembling a polysaccharide was precipitated, but the quantity was too small for further examination. No other product of interest was found in the mycelium.

B. *Treatment of Filtrate*.—Examination of the carbon balance sheet for this species indicates that a comparatively large yield (16 per cent.) of some volatile neutral body is to be expected, together with almost as large an amount (12 per cent.) of some product or products included in the balance sheet as "carbon unaccounted for." Hence a portion of the filtrate was distilled through a fractionating column and the distillate was refractionated. Two fractions of interest were obtained, the smaller one boiling below 77° C. and the main fraction boiling at 77°–80° C.

The fraction boiling below 77° C. gave a heavy yellow precipitate with 2:4-dinitrophenylhydrazine hydrochloride. This, on recrystallisation from absolute alcohol, had a melting-point of 158°–160° C., and corresponded with the 2:4-dinitrophenyl-

hydrazone of acetaldehyde, which according to BRADY and ELSMIE (1926), melts at 162° C.

A portion of the main fraction boiling at 77°–80° C. was treated with somewhat less than the theoretical amount of *p*-nitrobenzoyl chloride according to the method described by BUCHNER and MEISENHEIMER (1905). The mixture was warmed until complete solution was obtained and then cooled, when yellowish crystals separated. These were filtered, shaken with sodium carbonate solution to remove any free acid, filtered and dried. The material melted at 55°–56° C. and there was no depression of the melting point when mixed with an authentic specimen of ethyl *p*-nitrobenzoate. The main volatile neutral product is therefore ethyl alcohol, with small amounts of acetaldehyde.

The whole of the filtrate B was now evaporated *in vacuo* to low bulk and, on standing, became semi-solid and filled with crystals resembling mannitol. These crystals were not easily separated, however, so the whole residue was dissolved in water and precipitated with basic lead acetate, the lead precipitate (C) filtered off, excess lead removed from the filtrate by H₂S, lead sulphide filtered off and the filtrate evaporated *in vacuo* to a volume of 500 c.c.

One-half of this was diluted to one litre with water, 1 gm. of KH₂PO₄ added and the glucose present, amounting to about 60 gm., was removed by fermentation with a pure culture of yeast. At the end of the fermentation the yeast was filtered off, the filtrate treated with lead acetate, filtered from the lead precipitate which was rejected, and the lead removed from the filtrate with H₂S. The filtrate from the lead sulphide was evaporated *in vacuo* to remove H₂S, and made up to a volume of 500 c.c. Portions of this solution were now used for examination. 300 c.c. were evaporated to a syrup and treated while hot with sufficient absolute alcohol to produce incipient turbidity. On cooling, a mass of white needle crystals having a weight of 9.7 gm. = 3.23 per cent., separated. This was recrystallised and shown to consist of mannitol by the following means: A portion melted at 167°–168° C., the melting point being unchanged on admixture with pure mannitol. The optical rotation of another portion was $[\alpha]_{\text{Hg. yellow}}^{20} = -0.48^\circ$ in water ($c = 2.07$) changing to $[\alpha]_{\text{Hg. yellow}}^{20} = +35.2^\circ$ in 6 per cent. borax solution ($c = 0.829$). The value found in Part X for pure mannitol in 6 per cent. borax at a concentration of 0.829 per cent. was $+35.47^\circ$.

Experiments were now carried out to estimate the amount of mannitol present in the solution, with the following results:—

- (a) True mannitol by polarimeter in borax solution = 3.56 per cent.
- (b) "Mannitol" by acetylation with acetic anhydride and sodium acetate = 6.16 per cent.
- (c) "Mannitol" by estimation of total carbon in solution, after removal of the acetic acid arising from the lead acetate used in precipitation = 6.83 per cent.

Consideration of these figures makes it obvious that only about half of the organic material in solution is mannitol, and since the figures obtained in (b) and (c) are approximately equal, it is obvious that the other metabolic products must be fully hydroxylated. The alcoholic mother-liquors from the 300 c.c. portion from which 9.7 gm. of mannitol had been isolated were therefore evaporated to a syrup, taken up in absolute alcohol and 2 vols. of dry ether added. The ether-alcohol solution was filtered from a certain amount of sticky material which was precipitated and evaporated to a syrup which weighed 5.28 gm.

2.2 gm. of this syrup were now treated with benzoyl chloride according to the method of LIPP and MILLER (1913), for the preparation of glycerol tribenzoate. This method has proved the most useful of any described in this paper for the isolation and identification of glycerol.

3.39 gm. of glycerol tribenzoate were obtained, which on recrystallisation from absolute alcohol melted at 72° – 72.5° C., the melting point being unchanged on admixture with a sample of pure glycerol tribenzoate.

From the weight of glycerol tribenzoate isolated it is obvious that glycerol must be a metabolic product of this strain of *Helminthosporium*, since, while a little glycerol may have arisen during the fermentation of the residual sugar by yeast, as described on p. 333, the glycerol thus formed would be only a small proportion of the amount actually isolated.

The main metabolic products of this strain of *Helminthosporium geniculatum* when grown on glucose are thus ethyl alcohol and mannitol, together with smaller amounts of glycerol and acetaldehyde.

(b) *Clasterosporium species*.

Thirty litres of medium distributed between 6 trays were used in this experiment which commenced on 16th February, 1928. Aeration was commenced on 25th February, 1928. Each tray received 0.3 cu. ft. of air per day, 6 days per week, during the rest of the incubation period which, in all, lasted 32 days. Samples were taken from each tray on the 29th day and gave the following figures for glucose by the polarimeter:—0.53 per cent., 0.16 per cent., 1.14 per cent., 0.46 per cent., 0.45 per cent., 0.63 per cent., while an average sample gave 0.63 per cent. The experiment was stopped after 32 days, when all the six trays were found to be free from contamination. The metabolism solution was filtered from the mycelium, which was drained but not further examined, the volume of the solution measured and a well-mixed sample taken for analysis. The following results were obtained:—

Residual glucose:—(a) by polarimeter, 0.230 per cent.

(b) „ WOOD-OST, 0.244 „

(c) „ alkaline iodine, 0.544 per cent.

A partial carbon balance sheet prepared on the usual basis gave the results recorded in Table II.

TABLE II.

| | | | | | | Gm. Carbon per 250 c.c. Medium. |
|------------------------------------|-----|-----|-----|-----|-----|------------------------------------|
| | | | | | | Gm. |
| Carbon in residual glucose | ... | ... | ... | ... | ... | 0.244 |
| „ in CO ₂ in solution | ... | ... | ... | ... | ... | — |
| „ in volatile acids | ... | ... | ... | ... | ... | 0.025 |
| „ in non-volatile acids | ... | ... | ... | ... | ... | 0.086 |
| „ in volatile neutral compounds... | ... | ... | ... | ... | ... | 0.824 |
| „ in synthetic carbon | ... | ... | ... | ... | ... | Nil |
| Total carbon accounted for | ... | ... | ... | ... | ... | 1.179 |
| „ „ in solution | ... | ... | ... | ... | ... | 2.181 |
| Carbon unaccounted for | ... | ... | ... | ... | ... | 1.002 |

Treatment of Metabolism Solution.—The results given in Table II are in substantial agreement with those previously recorded for the same species. They indicate that about 16 per cent. of some volatile neutral compound is to be expected, together with 20 per cent. of some product or products included in the balance sheet as “carbon unaccounted for.” Hence the metabolism solution which, after the removal of the portion for analysis, measured 22.2 litres, was evaporated *in vacuo*. The first portions of the vacuum distillate were then fractionated at ordinary pressure through a YOUNG’S column. There was thus obtained 45 c.c. of a liquid boiling at 77° C. to 79° C., which was shown to consist of ethyl alcohol, containing small amounts of acetaldehyde, by the method described on p. 332. Thus 2:4-dinitrophenylhydrazine gave rise to a hydrazone which on recrystallisation sintered at 160° C. and melted at 162°–162.5° C., while *p*-nitrobenzoyl chloride gave a nitrobenzoate which melted at 54.7°–56° C.

The whole of the metabolism solution which had now been evaporated to about 5 litres was not fermented by yeast, but was treated direct with basic lead acetate solution until no further precipitate was obtained and the solution (A) filtered from the lead precipitate (B), treatment of which will be given later.

Treatment of solution A.—Lead was removed from the solution (A) by H₂S and half of this lead-free solution was evaporated to a syrup which was then treated with hot absolute alcohol. On cooling, a considerable quantity of crystalline material separated, having a weight of 68.3 gm. (Fraction 1). This was filtered off, washed with absolute alcohol and shown to be mannitol by the method given on page 333.

The alcoholic mother-liquors were shown by analysis to contain a further 16.0 gm. of mannitol, which with Fraction 2, 1.7 gm. (p. 336) gives a total of 86 gm. equivalent to 172 gm. in the original volume. By treatment of the alcoholic mother-liquors as described on p. 334, 5.66 gm. of glycerol tribenzoate (M.Pt. 71°–72° C.) were isolated.

Treatment of lead precipitate (B).—The lead precipitate referred to on p. 335 was ground up with excess of dilute sulphuric acid. The lead sulphate formed was filtered off and the filtrate, freed from sulphuric acid by means of barium hydroxide, and from lead by means of sulphuretted hydrogen, was evaporated *in vacuo* to a thick syrup. The acids in this syrup were esterified by boiling with three separate portions of 250 c.c. of absolute ethyl alcohol containing 2·5 per cent. HCl. There separated from the acid esterification mixture a small amount of crystalline material which weighed 1·7 gm., was shown to be mannitol, and constituted Fraction 2. The filtrate from this fraction, after removal of alcohol, was extracted with ether, the ether extract washed with sodium carbonate and water, dried over anhydrous sodium sulphate and the esters distilled *in vacuo*. Two main fractions were obtained. Fraction 1 distilled at 106° C. to 130° C. at 18 mm. and weighed 7·8 gm. Fraction 2 distilled at 135° C. to 161° C. at 18 mm. and weighed 3·3 gm. 1 c.c. of the first fraction, on treatment with 2 c.c. of absolute alcohol and 2 c.c. of 50 per cent. hydrazine hydrate, gave a copious amount of a crystalline hydrazide which on recrystallisation separated in plates melting at 164°–165° C. This was shown to be succinic acid dihydrazide which has the same melting point. A mixed melting point gave 165° C. Fraction 2 on similar treatment with hydrazine hydrate gave rise to a small amount of a crystalline hydrazide melting at 176°–177° C. which was probably malic acid dihydrazide.

The main metabolic products of this species of *Clasterosporium*, when grown on glucose, are thus mannitol and ethyl alcohol, together with smaller amounts of glycerol, acetaldehyde, succinic acid and probably malic acid.

(c) *Aspergillus Wentii*.

Sixty litres of medium distributed between twelve trays were used in this experiment which commenced on 10th June, 1927. Aeration was commenced on 15th June, 1927, each tray receiving 0·3 cu. ft. daily, six days per week, during the rest of the incubation period, which terminated on 22nd July, 1927. Samples were taken periodically in order to follow the course of the fermentation, and the results are given in Table III, since they illustrate very effectively a point which is evident from the original carbon balance sheet, *i.e.*, the production of some optically dextro-rotatory material which does not reduce alkaline copper solution. This is indicated by the large difference found between the glucose as estimated by the polarimeter and by the copper reduction method of Wood-Ost. (Table III.)

The experiment was terminated after 42 days' incubation. There was no contamination in any of the twelve trays, so that the whole of the metabolism solution from all the trays was mixed and filtered from the mycelium, which was pressed out, but not investigated further. The filtered metabolism solution had a total volume of 45·10 litres, of which 450 c.c. were taken for analysis and the remaining 44·65 litres were evaporated to low

Table III.—Percentage of glucose in different samples as estimated (a) Polarimetrically ;
(b) by the WOOD-OST Method.

| Number of Tray. | After 17 Days' Incubation. | | After 24 Days' Incubation. | | After 35 Days' Incubation. | | After 42 Days' Incubation. | |
|--------------------|-------------------------------|------------------|-------------------------------|------------------|-------------------------------|------------------|-------------------------------|------------------|
| | By Polari- meter. | By WOOD- OST. | By Polari- meter. | By WOOD- OST. | By Polari- meter. | By WOOD- OST. | By Polari- meter. | By WOOD- OST. |
| 2 | 2.77 | 2.26 | 2.19 | 1.67 | 1.21 | 0.61 | — | — |
| 4 | 3.26 | 2.70 | 2.49 | 1.97 | 1.52 | 0.95 | — | — |
| 9 | 2.98 | 2.50 | 2.27 | 1.74 | 1.02 | 0.53 | — | — |
| 11 | 2.90 | 2.48 | 2.09 | 1.61 | 0.90 | 0.38 | — | — |
| Average sample | — | — | — | — | — | — | 0.64 | 0.25 |

bulk *in vacuo*. A partial balance sheet prepared on the sample taken for analysis gave the following results :—

Titration value, 14.3 c.c. of N/1 NaOH per 250 c.c. medium.

Glucose by polarimeter, 0.641 per cent.

Glucose by WOOD-OST, 0.250 per cent.

Treatment of Metabolism Solution.

The results given in Table IV indicate that *Aspergillus Wentii* produces no alcohol, but, on the other hand, gives rise to about 20 per cent. of some non-volatile acid, the calcium salt of which is precipitated by 80 per cent. alcohol, together with about 16 per cent. of some product included as "carbon unaccounted for." The whole of the

Table IV.

| | | | | | | | Gm. Carbon per 250 c.c. Medium. |
|------------------------------------|-----|-----|-----|-----|-----|-----|------------------------------------|
| | | | | | | | Gm. |
| Carbon in residual glucose | ... | ... | ... | ... | ... | ... | 0.250 |
| " in CO ₂ in solution | ... | ... | ... | ... | ... | ... | 0.006 |
| " in volatile acids | ... | ... | ... | ... | ... | ... | 0.015 |
| " in non-volatile acids | ... | ... | ... | ... | ... | ... | 0.991 |
| " in volatile neutral compounds... | ... | ... | ... | ... | ... | ... | Nil |
| " in synthetic carbon | ... | ... | ... | ... | ... | ... | 0.067 |
| Total carbon accounted for | ... | ... | ... | ... | ... | ... | 1.329 |
| " " in solution | ... | ... | ... | ... | ... | ... | 2.141 |
| Carbon unaccounted for... | ... | ... | ... | ... | ... | ... | 0.812 |

metabolism solution was therefore evaporated *in vacuo* to about 9 litres. Of this, two-thirds was stored in the cold room under toluene, while *one-third* was completely precipitated with basic lead acetate. There was a very large precipitate of lead salts (A), which was filtered off and washed well with water. The treatment of the filtrate and washings (B) will be described later.

A. *Treatment of Lead Precipitate*.—The acids were regenerated from the lead precipitate by treatment with dilute sulphuric acid in the usual manner, excess of sulphuric acid being removed from the filtrate quantitatively by means of barium hydroxide. The solution of regenerated acids was now maintained at 90°–95° C. in presence of an excess of calcium carbonate. When the solution was apparently neutral to litmus, calcium hydroxide was added until a permanently neutral solution was obtained, in order to convert any lactone present into the free acid. The solution of the calcium salts was filtered from the excess of calcium carbonate, which was carefully washed, and the filtrate and washings evaporated *in vacuo* to incipient crystallisation. On standing in the cold, large quantities of a crystalline calcium salt were deposited, which were removed from time to time. In all, four crops were obtained having a total weight of 191.6 gm. This crystalline calcium salt consisted of practically pure calcium gluconate, which it resembles in crystalline form and in its characteristic method of crystallisation. For identification, a portion of it was converted into the phenylhydrazide by heating on a water bath with phenylhydrazine and acetic acid. The phenylhydrazide was recrystallised from water and decolorised with a little animal charcoal. It separated in shining white plates having a melting point of 204°–205° C., which was unchanged on admixture with synthetic gluconic acid phenylhydrazide. Another portion of the calcium salt was recrystallised from water and the calcium gluconate content determined by:—

- | | |
|---|------------------|
| (a) determination of optical activity using the figure given by MAY, HERRICK, THOM and CHURCH (1927), of $[\alpha]_D^{20} = +9.8^\circ$ | = 95.8 per cent. |
| (b) ashing in presence of sulphuric acid | = 95.1 „ |

It may be noted here that 191.6 gm. of calcium gluconate were actually isolated from *one-third* of the metabolism solution, so that it seems reasonable to suppose that the amount of calcium gluconate actually present is considerably higher than this. On the other hand, if it is assumed that *all* the carbon present in the balance sheet in Table IV as non-volatile acids is present as gluconic acid, only 176.2 gm. of calcium gluconate should be present in this volume of metabolism solution. The explanation of this discrepancy is almost certainly the fact that gluconic acid is present in the original metabolism solution, partly as the free acid, which is then precipitated as the calcium salt in 80 per cent. alcohol, and partly as the lactone, which is not precipitated as the calcium salt, but which is shown as “carbon unaccounted for.” Further support for this view will be given later, when it is shown that, in spite of the high

figure for "carbon unaccounted for," only relatively small amounts of products other than gluconic acid could be isolated.

B. *Treatment of Filtrate and Washings*.—The filtrate and washings were freed from lead by means of H_2S and then evaporated *in vacuo* to a syrup which did not crystallise even after long standing. It was, therefore, re-dissolved in 1 litre of water and the residual glucose, amounting to about 37 gm., fermented with 40 gm. of pure yeast. After three days the yeast was removed by filtration, the filtrate cleaned with basic lead acetate, and the filtrate from the lead precipitate freed from lead by means of H_2S and then evaporated *in vacuo*. Even now the resultant syrup refused to crystallise, and the explanation was found in the fact that the syrup contained considerable quantities of sodium acetate. This was removed by estimating the acetic acid in a small portion of the syrup and then distilling the remainder *in vacuo* with the calculated amount of sulphuric acid necessary to decompose the sodium acetate present. After removal of the acetic acid, an estimation of the carbon present in solution gave an amount only half (24.8 gm.) of what was expected from the balance sheet, *i.e.*, 48.6 gm. of carbon should be present as "carbon unaccounted for" in one-third of the metabolism solution. This supports the view already expressed that part of the "carbon unaccounted for" is gluconic acid lactone, which by the method adopted will have been isolated as calcium gluconate.

The acetic acid-free solution was now evaporated, when considerable quantities of pure sodium sulphate were separated. The resulting syrup was then fractionally precipitated with alcohol and finally with alcohol and ether, giving rise to

- (a) a series of alcohol and ether-alcohol precipitates,
- (b) an ether-alcohol solution.

(a) *Treatment of Alcohol and Ether-Alcohol Precipitates*.—The following fractions were obtained after treatment with alcohol and ether-alcohol:—

TABLE V.

| Fraction. | Treatment. | Weight in Gm. | Percentage of Sulphated Ash. |
|-----------|---|---------------|------------------------------|
| 1 | Crystalline magma from 70 per cent. alcohol ... | 9.73 | 32.4 |
| 2 | Immediate precipitate from 95 per cent. alcohol ... | 12.6 | 9.07 |
| 3 | Precipitate from 95 per cent. alcohol on standing ... | 2.51 | 0.85 |
| 4 | Precipitate from 1 : 1 ether-alcohol | 6.84 | 5.43 |
| 5 | Precipitate from 2 : 1 ether-alcohol | 1.32 | 4.04 |
| 6 | Syrupy residue | 16.0 | 0.13 |

Apart from a little mineral matter, as indicated by the figures for sulphated ash, fractions 2–5 consisted almost entirely of mannitol. Thus, a portion of fraction 3

crystallised from water and alcohol in the manner characteristic of mannitol, in needles having a melting point of 164.5° – 166° C., which was not altered on admixture with a sample of pure mannitol. Actual estimations of the amount of mannitol present were carried out by the method described in Part X, and indicated the presence of a total of 28.0 gm. of mannitol in one-third of the total metabolism solution.

(b) *Treatment of Ether-alcohol Solution.*—The ether-alcohol solution on evaporation left 16 gm. of a syrup referred to as fraction 6 in Table V. A portion of this syrup was very carefully dried *in vacuo*, when it lost 1–2 per cent. of its weight. 6.3 gm. of this dried syrup were treated with 26 gm. of phenyl isocyanate and the mixture warmed on a sand bath under a reflux condenser until the reaction commenced, when the source of heat was immediately removed until the initial violent reaction had moderated. Heating was then resumed for a further 20 minutes. The product, which solidified on cooling, was broken up, washed with cold benzene and then with water, and recrystallised from alcohol. The solid residue was then fractionally recrystallised from alcohol and benzene, and after 28 separate recrystallisations the purified products were found to group themselves around two melting points, *i.e.*, 183° – 185° C. and 235° C., the latter corresponding to diphenyl urea.

The fraction melting at 183° – 185° C. was shown to be the triphenylurethane of glycerol. Its melting point was not depressed on admixture with a synthetic sample of the triphenylurethane of glycerol, with which it also agrees in appearance and crystalline form. The melting point which was found for this material differs from the only melting point given in the literature, *i.e.*, 160° C. to 180° C. of TESSMER (1885). A combustion on the material melting at 183° C. to 185° C. gave the following results:—

0.1295 gm. of urethane gave 0.0590 gm. H_2O and 0.3055 gm. CO_2 , corresponding to 5.10 per cent. hydrogen and 64.33 per cent. carbon.

0.1161 gm. gave 0.0561 gm. H_2O and 0.2755 gm. CO_2 , corresponding to 5.41 per cent. hydrogen and 64.70 per cent. carbon.

0.1428 gm. gave 11.02 c.c. nitrogen at 13.5° C. and 774.1 mm., corresponding to 9.27 per cent. nitrogen.

The calculated results for glycerol triphenylurethane ($\text{C}_{24}\text{H}_{23}\text{O}_6\text{N}_3$) are: carbon = 64.15 per cent., hydrogen = 5.16 per cent., nitrogen = 9.35 per cent.

The total yield of glycerol triphenylurethane isolated was found to correspond to over 50 per cent. of the theoretical, assuming that the original syrup consisted entirely of glycerol. Since no trace of any other phenylurethane could be isolated, and making due allowance for the known losses arising during recrystallisation, the syrup must, obviously, consist mainly of glycerol.

The objection might be raised that part, at any rate, of this glycerol had arisen during the removal of residual sugar by fermentation with yeast, as described on p. 339. For this reason a second portion, consisting of one-third of the original evaporated metabolism solution, was worked up in the same manner as has already been described, with the exception that the residual glucose was not removed by fermentation, but was

precipitated with alcohol and ether in the mannitol fraction. By this means a syrup weighing 20.2 gm. was obtained which on analysis gave 75.3 per cent. and 75.6 per cent. of glycerol by the pyridine acetylation method and 76.2 per cent. by the ZEISEL-FANTO method. It is thus evident that glycerol is really a metabolic product of *Aspergillus Wentii*.

The main product of the metabolism of *Aspergillus Wentii* when grown on glucose is thus gluconic acid (about 16 per cent. isolated) which is present in the metabolism solution partly as the free acid, the calcium salt of which is precipitated by 80 per cent. alcohol, and partly as the lactone which in the metabolism experiments appears in the fraction "carbon unaccounted for." Mannitol (2.9 per cent.) and glycerol (1.6 per cent.) are the other main metabolic products, while no alcohol is formed by this species.

(d) *Fumago vagans*.

Sixty litres of medium distributed between 12 trays were used in this experiment which commenced on 6th October, 1928. Aeration was commenced on 13th October, 1928, each tray receiving 0.2 cu. ft. per day, six days per week. Eight trays were taken off on 13th November, 1928, while the remaining four, which were somewhat slower, were taken off on 19th November, 1928.

Samples were taken and analysed periodically and a selection of the results is given in Table VI. These results indicate the production of some optically dextro-rotatory material which does not reduce alkaline copper solution. The production of a large amount of titratable acid is also a noteworthy feature. During the estimation of the titratable acid it was always found that the initial end-point was very transitory and that it was necessary to add considerable volumes of standard sodium hydroxide after the apparent end-point has been reached before a really permanent and final pink colour was obtained. The same feature was noted with *Aspergillus Wentii* and is probably due to the presence of gluconic acid lactone.

Eight trays were taken off after 38 days' incubation and the remaining four after 44 days. All the trays were entirely free from contamination so that the whole of the metabolism solution (A) from all the trays was mixed and filtered from the mycelium (B). The filtered metabolism solution had a total volume of 44.49 litres. 100 c.c. were taken for analysis and gave the following analytical results:—

Titration value = 26.2 c.c. N/1 NaOH per 250 c.c. medium.

Glucose by polarimeter = 0.874 per cent.

Glucose by WOOD-OST = 0.21 per cent.

A carbon balance sheet was not prepared for this particular sample, but a balance sheet prepared on a repeat experiment, which followed almost identical lines, is appended. (Table VII.)

TABLE VI.—Showing (a) Per cent. Glucose estimated polarimetrically; (b) Per cent. Glucose estimated by Wood-Ost Method, and (c) Titration in c.c. N/1 NaOH per 250 c.c. solution.

| Tray No. | After 20 Days' Incubation. | | | After 27 Days' Incubation. | | | After 33 Days' Incubation. | | | After 37 Days' Incubation. | | | Glucose by Wood-Ost. | Titration in c.c. |
|----------------|----------------------------|----------------------|-------------------|----------------------------|----------------------|-------------------|----------------------------|----------------------|-------------------|----------------------------|----------------------|-------------------|----------------------|-------------------|
| | Glucose by Polari-meter. | Glucose by Wood-Ost. | Titration in c.c. | Glucose by Polari-meter. | Glucose by Wood-Ost. | Titration in c.c. | Glucose by Polari-meter. | Glucose by Wood-Ost. | Titration in c.c. | Glucose by Polari-meter. | Glucose by Wood-Ost. | Titration in c.c. | | |
| 2 | 2.11 | 1.77 | 23.6 | 1.47 | 1.00 | 27.9 | 1.05 | 0.51 | 30.7 | 0.86 | — | 30.5 | — | — |
| 10 | 2.33 | 1.66 | 26.0 | 1.71 | 0.91 | 30.5 | 1.34 | 0.50 | 32.7 | 1.18 | — | 32.7 | — | — |
| Average Sample | — | — | — | — | — | — | — | — | — | — | 0.874 | — | 0.210 | 26.2 |

TABLE VII.

| | | | | | | Gm. Carbon per 250 c.c. Medium. |
|--|-----|-----|-----|-----|-----|------------------------------------|
| | | | | | | Gm. |
| Carbon in residual glucose | ... | ... | ... | ... | ... | 0.345 |
| „ in CO ₂ in solution | ... | ... | ... | ... | ... | Nil |
| „ in volatile acids | ... | ... | ... | ... | ... | — |
| „ in non-volatile acids | ... | ... | ... | ... | ... | 1.198 |
| „ in volatile neutral compounds | ... | ... | ... | ... | ... | Nil |
| „ in synthetic carbon | ... | ... | ... | ... | ... | 0.017 |
| „ in compounds precipitated by alcohol | ... | ... | ... | ... | ... | 0.211 |
| Total carbon accounted for | ... | ... | ... | ... | ... | 1.771 |
| „ „ in solution | ... | ... | ... | ... | ... | 2.521 |
| Carbon unaccounted for | ... | ... | ... | ... | ... | 0.750 |

It will be noticed that one of the items in the carbon balance sheet, *i.e.*, “carbon in compounds precipitated by alcohol” = 0.211 gm., has not hitherto appeared in the carbon balance sheets. It was found, however, in dealing with *Fumago vagans* that a heavy precipitate was formed on the addition of four volumes of alcohol to the metabolism solution on which the estimation of the carbon in non-volatile acids was being carried out. This precipitate was therefore filtered off, and its carbon content estimated. The calcium salts of the non-volatile acids were then precipitated in the filtrate. The nature of this alcohol insoluble precipitate (C) will be dealt with later.

A. *Treatment of metabolism solution.*—The results given in Table VII indicate that *Fumago vagans* produces no alcohol, but, on the other hand, gives rise to large amounts of non-volatile acids (24 per cent.) together with about 15 per cent. of “carbon unaccounted for” and about 4 per cent. of some product which is precipitated from aqueous solution by the addition of four volumes of alcohol. Hence, the whole of the metabolism solution was evaporated *in vacuo* to about 3 litres and of this *one-half*, 1560 c.c., was taken for further treatment. An equal volume of 66 O.P. alcohol was added very slowly with continuous stirring, causing the formation of a voluminous precipitate which was allowed to settle overnight. This precipitate, which was neutral in reaction and which constitutes the alcohol insoluble precipitate (C), was filtered off, and washed with 50 per cent. alcohol.

The filtrate and washings were evaporated *in vacuo* to about 700 c.c. and, since a test carried out on a small portion of this solution indicated the presence of considerable amounts of gluconic acid, an attempt was made to fractionate whatever acids were present. This was carried out as follows: To the evaporated and strongly acid filtrate were added two volumes of alcohol with constant stirring. A strongly acid syrup weighing 157 gm. was obtained and this was dissolved in water, neutralised with sodium hydroxide

and treated with a solution of calcium acetate in water. The calcium salts were then fractionally precipitated with alcohol giving rise to Fractions B1 to B5.

The filtrate from the above syrup was now neutralised, enough alcohol added to give 80 per cent. by volume, and calcium acetate added gradually and with constant stirring. A very voluminous precipitate was obtained which was filtered off, washed and fractionally crystallised from water. This gave rise to Fractions C1 to C8. The alcoholic filtrate from the above calcium salts insoluble in 80 per cent. alcohol was evaporated to remove all the alcohol and then set aside to crystallise. This gave rise to Fractions D1 to D3. Treatment of the mother-liquors from D1 and D3 will be described later.

Each of the different fractions B1 to B5, C1 to C8 and D1 to D3 was now recrystallised from water and the mother-liquors from the recrystallisations used for the preparation of lead salts, as described later. The different fractions were then analysed by the following method which will indicate the presence of any material other than calcium gluconate. The calcium gluconate content of the fraction under examination was first determined by the polarimeter using $[\alpha]_D^{20} = +9.8^\circ$ (MAY, HERRICK, THOM and CHURCH, 1927) as the accepted figure for pure calcium gluconate. The calcium gluconate in the same sample was then estimated by determining the sulphated ash.

The quotient $R \equiv \frac{\text{calcium gluconate by polarimeter}}{\text{calcium gluconate by sulphated ash}}$ is, of course, unity in the case of pure calcium gluconate. The value obtained for each of the different fractions, together with their weights, is given in Table VIII.

The figures given for R in Table VIII show a remarkable approximation to unity considering the range of fractions, and indicate that almost all the fractions consist of fairly pure calcium gluconate. Two of the fractions were examined for the presence of other acids, *i.e.*, fraction C4, having a value for $R = 0.944$ and fraction D3, having a value for $R = 0.482$. 10 gm. of fraction C4 were dissolved in water, the calcium removed with oxalic acid, filtered, the filtrate evaporated and extracted with ether. A small amount of crystals of succinic acid was obtained. Fraction D3, on similar treatment, gave a very strong smell of acetic acid, and it is evident that this fraction contained a considerable amount of calcium acetate, which would explain the low value for R.

An attempt was also made to detect other acids in the mother-liquors from the recrystallisation of the calcium salts referred to above. The mother-liquors from all the calcium salt recrystallisations were combined and treated with the theoretical amount of oxalic acid to precipitate the amount of calcium present, filtered from the calcium oxalate and the filtrate precipitated with basic lead acetate. The lead salts were then filtered, washed and dried. 202 gm. of dry lead salts were obtained, and these were converted into esters by FOREMAN'S method (1912), involving esterification with absolute ethyl alcohol and dry HCl, filtration from precipitated lead chloride and removal of excess of HCl as NH_4Cl by treatment with a solution of dry NH_3 in absolute alcohol. The alcoholic solution of the esters when

TABLE VIII.

| Number of Fraction. | Weight of Fraction in Gm. | Value of R |
|-----------------------------------|--|---|
| | | $= \frac{\text{Calcium Gluconate by Polarimeter.}}{\text{Calcium Gluconate by Sulphated Ash.}}$ |
| B 1 | 6.76 | 0.986 |
| B 2 | 15.15 | 0.966 |
| B 3 | 1.33 | 1.060 |
| B 4 | 36.69 | 1.049 |
| B 5 | 5.17 | 0.925 |
| C 1 | 10.23 | 0.988 |
| C 2 | 17.68 | 0.962 |
| C 3 | 87.50 | 0.998 |
| C 4 | 32.09 | 0.944 |
| C 5 | 6.87 | 0.985 |
| C 6 | 17.95 | 0.920 |
| C 7 | 29.60 | 0.938 |
| C 8 | 41.90 | 0.913 |
| D 1 | 13.12 | 0.957 |
| D 2 | 3.73 | 1.16 |
| D 3 | 12.87 | 0.482 |
| Theoretical for calcium gluconate | — | 1.000 |
| — | Total weight of different fractions = 338.64 | — |

freed from HCl was evaporated to remove all alcohol, and then shaken with dry ether. The ether solution was dried over Na_2SO_4 , the ether removed and the residue, which was very small, distilled *in vacuo*. Only 2 to 3 drops of an oily liquid distilled, having a boiling point about 130°C . at 4 to 5 mm., nor could any distillable esters be obtained from the ether insoluble part, even though an attempt was made to distil the esters in a mercury vapour vacuum. It is thus apparent that the mother-liquors contain practically no acids giving esters which can be distilled, and it appears probable that no acid other than gluconic acid is present in any appreciable amount.

The mother-liquors from fractions D1 to D3 of the calcium salts contain the whole of the metabolic products in solution, except such as were precipitated with 50 per cent. alcohol, *i.e.*, the alcohol insoluble precipitate C, together with those acids precipitated as calcium salts in 80 per cent. alcohol. For further investigation, these mother-liquors were therefore evaporated to small bulk, and sufficient 5N. H_2SO_4 added (220 c.c.) to give an acid reaction to congo red. The precipitated calcium sulphate was filtered off and the filtrate extracted with ether in a continuous extraction apparatus. 11.6 gm. of a crystalline product were obtained on evaporation of the ether, and this was found to consist of succinic acid. On recrystallisation from alcohol and sublimation in a

high vacuum, this product melted at 192° – 193° C., and this melting point was not altered on admixture with an authentic sample of succinic acid. 0.2426 gm. required 40.96 c.c. of N/10 NaOH for neutralisation corresponding to an equivalent of 59.24 (theoretical for succinic acid = 59.02).

The ether extracted solution was neutralised and evaporated to small bulk. It was now mixed with wood meal in order to convert it into a crumbly mass suitable for extraction, and was then dried down *in vacuo* to a friable mass, which was extracted with acetone in a continuous extraction apparatus. The acetone solution was evaporated, the residue taken up in absolute alcohol and the alcoholic solution treated with 2 volumes of dry ether. The ether-alcohol solution was filtered and evaporated, leaving 10.73 gm. of syrup, which was shown to consist principally of glycerol. 1 gm. of this syrup on treatment with benzoyl chloride, according to the method of LIPP and MILLER (1913) gave 2.65 gm. of crude glycerol tribenzoate, which on recrystallising twice from absolute alcohol, melted at 71.5° – 72° C., the melting point being unchanged on admixture with a sample of pure glycerol tribenzoate. This weight of glycerol tribenzoate corresponds to about 60 per cent. of pure glycerol in the syrup. An estimation of the glycerol content by oxidation with periodic acid according to the method of MALAPRADE (1928) gave the following results: glycerol by the iodine absorbed = 69.9 per cent., and glycerol by the acidity formed = 68.9 per cent. Since the agreement between these two methods is good, it may be assumed that no product susceptible to oxidation by periodic acid is present in the syrup other than glycerol.

At no time was there any evidence of the presence of mannitol in the metabolism solution.

B. *Treatment of Mycelium*.—The mycelium (see p. 341) was treated with about 2 litres of distilled water, steamed, and pressed out while still hot, giving rise to a sticky solution. This extraction was repeated four times in all, and to the combined filtered extracts was now added an equal volume of alcohol. This gave rise to a voluminous precipitate, which weighed 8.2 gm., and which was identical with the "alcohol precipitate C," referred to on p. 343, and isolated from the metabolism solution. The two fractions were therefore combined for further treatment.

C. *Treatment of Alcohol Insoluble Precipitate*.—The alcohol insoluble precipitate C obtained from the metabolism solution weighed 24.5 gm., and was in the form of a greyish powder. This was combined with the same product obtained from the mycelium (wt. = 8.2 gm.), and a portion of it was purified by fractional precipitation from aqueous solution with alcohol. By this means the dark colouring matter present was precipitated in the first fractions, and the material arising in the middle fractions consisted of a white flocculent precipitate, which when washed with alcohol and dry ether, and then quickly dried *in vacuo* gave rise to a light white powder. This purified material was examined, and was shown to be of the nature of a neutral polysaccharide, which is apparently different from any previously described.

General Properties of the Alcohol Insoluble Precipitate.—This material is readily soluble in hot water, from which it slowly separates, on cooling, as a white amorphous precipitate. The aqueous solution is neutral to litmus, and gives no colour with iodine.

A portion of it gave the following results on combustion, corresponding to the empirical formula $(C_6H_{10}O_5)_n$: 0.2483 gm. of material dried *in vacuo* to constant weight gave 0.4031 gm. CO_2 and 0.1456 gm. H_2O corresponding to 44.27 per cent. of carbon and 6.56 per cent. of hydrogen (theoretical for $C_6H_{10}O_5$, carbon = 44.42 per cent., hydrogen = 6.22 per cent.).

It is very strongly dextro-rotatory. A 1.099 per cent. solution had a rotation corresponding to $[\alpha]_{D}^{18} = +217^\circ$.

It is readily hydrolysed by boiling with dilute acids and on hydrolysis gives rise to no product other than glucose. This was shown as follows:—1.0985 gm. of purified and dried material containing 0.0025 gm. of ash, and equivalent, therefore, to 1.096 gm. of dry ash-free material, were dissolved in water and made up to 100 c.c. 50.00 c.c. of this solution were now taken, 10 c.c. of N/1 HCl added, boiled under reflux and the rotation determined at intervals with the following results:—The readings were taken in the mercury yellow light in a 10 cm. tube. Initial reading, $+1.98^\circ$; after $2\frac{1}{4}$ hours' hydrolysis, $+0.61^\circ$; after $3\frac{3}{4}$ hours', $+0.49^\circ$; after $5\frac{3}{4}$ hours' $+0.48^\circ$.

The hydrolysis was stopped after $5\frac{3}{4}$ hours and 50.02 c.c. of the hydrolysis solution were removed and titrated with N/1 NaOH. 8.41 c.c. were required, and since the calculated amount of acid present equals $5/6 \times 10.00 = 8.33$ c.c., it is evident that no acid has been formed during the hydrolysis.

The neutralised solution was now made up to 100 c.c., and the glucose content estimated with the following results:—

| | Per cent. |
|--------------------------------|-----------|
| (a) By polarimeter | = 0.474 |
| (b) By Wood-Ost | = 0.475 |
| (c) By alkaline iodine | = 0.489 |

Accepting the polarimeter and Wood-Ost figures as being correct, the actual weight of glucose produced from 1.096 gm. of dry ash-free material is thus 1.140 gm., from which it is obvious that no product other than glucose is formed during the hydrolysis.

It was definitely shown that the hydrolysis product is glucose, and not some other carbohydrate, by hydrolysing 2 gm. of material with 50 c.c. of N/10 HCl for 5 hours. 5 c.c. of N/1 NaOH were added to neutralise the acid, then 6 c.c. of phenylhydrazine and 3 c.c. of glacial acetic acid. The mixture was heated on the boiling water bath for half an hour, and the osazone which separated in yellow needles was filtered off, washed and dried. The dry osazone melted at $206^\circ C$. when quickly heated and 0.2 gm. dissolved in 4 c.c. of pyridine and 6 c.c. of alcohol and polarised in a 10 cm. tube gave a

rotation of -1.35° and -1.45° on different fractions. NEUBERG (1899) gives the value for glucose under these conditions as -1.50° .

The polysaccharide is not hydrolysed by either of the enzymes, invertase or diastase, since solutions of the polysaccharide to which solutions of the enzymes had been added showed no alteration in rotation even after 3 days' incubation at 37°C .

The main product of the metabolism of *Fumago vagans* when grown on glucose is thus gluconic acid. A new dextro-rotatory polysaccharide, composed entirely of glucose units, is also formed in considerable amounts. Glycerol and succinic acid are also formed in smaller amounts, but no mannitol was isolated.

(e) *Penicillium species* Ad. 11 (*P. chrysogenum*).

Sixty litres of medium distributed between 12 trays were used in this experiment, which commenced on 19th May, 1927. Aeration was commenced on 23rd May, 1927, each tray receiving 0.33 cu. ft. per day on 6 days per week. A sample of gas taken on 27th May, 1927, before aeration, showed an entire absence of oxygen and the presence of 21 per cent. of CO_2 . Four trays were taken off on 22nd June, 1927, and the remaining 8 trays were taken off 2 days later. All the trays were free from infection, so that the metabolism solution from all the trays was mixed and filtered, and had a total volume of 45 litres. Analyses are given in Table IX on all the trays on the day they were taken off.

TABLE IX.

| Number of Tray. | Titration in c.c. N/1 NaOH per 250 c.c. Medium. | Percentage Glucose by Polarimeter. | Percentage Glucose by Wood-Ost. |
|-----------------|---|------------------------------------|---------------------------------|
| 1 | 17.2 | 0.56 | 0.07 |
| 2 | 19.2 | 0.67 | 0.18 |
| 3 | 16.5 | 0.75 | 0.29 |
| 4 | 16.1 | 0.71 | 0.25 |
| 5 | 17.1 | 0.66 | 0.20 |
| 6 | 16.6 | 0.59 | 0.17 |
| 7 | 19.6 | 0.61 | 0.12 |
| 8 | 22.0 | 0.32 | 0.10 |
| 9 | 19.7 | 0.66 | 0.15 |
| 10 | 17.6 | 0.74 | 0.20 |
| 11 | 19.9 | 0.70 | 0.16 |
| 12 | 20.1 | 0.67 | 0.14 |

It was noted on carrying out the estimation of the titratable acidity that the initial end-point was very transitory, and that a considerable quantity of standard alkali was required to effect a permanent end-point. This is almost certainly due to the presence of an acid lactone.

A carbon balance sheet was prepared on a mixed sample obtained by taking 20 c.c. from each tray. The results are given in Table X.

| | | | |
|------------------------|----|----|---|
| Titration value | .. | .. | 20.3 c.c. N/1 NaOH per 250 c.c. medium. |
| Glucose by polarimeter | .. | | 0.552 per cent. |
| Glucose by WOOD-OST | .. | | 0.122 per cent. |

TABLE X.

| | | | | | Gm. Carbon per 250 c.c. Medium. |
|----------------------------------|-----|-----|-----|-----|------------------------------------|
| | | | | | Gm. |
| Carbon in residual glucose | ... | ... | ... | ... | 0.122 |
| „ in CO ₂ in solution | ... | ... | ... | ... | — |
| „ in volatile acids | ... | ... | ... | ... | — |
| „ in non-volatile acids | ... | ... | ... | ... | 0.833 |
| „ in volatile neutral compounds | ... | ... | ... | ... | Nil |
| „ in synthetic carbon | ... | ... | ... | ... | 0.106 |
| Total carbon accounted for | ... | ... | ... | ... | 1.061 |
| „ „ in solution | ... | ... | ... | ... | 2.472 |
| Carbon unaccounted for | ... | ... | ... | ... | 1.411 |

Treatment of Metabolism Solution.—The results given in Table X indicate that this strain of *P. chrysogenum* gives rise to about 16 per cent. of some non-volatile acid, the calcium salt of which is precipitated by 80 per cent. alcohol. There is also about 28 per cent. of some compound or compounds included as “carbon unaccounted for,” and in view of previous experience it seems probable that part, at any rate, of the “carbon unaccounted for” may be present as the lactone of a hydroxy-acid.

The whole of the metabolism solution, measuring 45 litres, was evaporated *in vacuo* to a thin syrup measuring 1,770 c.c. (solution A). This was preserved with toluene, and portions of it were examined.

500 c.c. of the original solution A (measuring 1,770 c.c.) were diluted to 3½ to 4 litres, and incubated at 30° C. for 24 hours with 22 gm. of pure yeast in order to remove all fermentable sugar. The solution was now filtered from the yeast, and the filtrate and washings precipitated with basic lead acetate solution until no further precipitate was obtained and the reaction of the liquid was definitely alkaline. The large lead precipitate was filtered off and washed by grinding three times with water. The filtrate and washings were combined, evaporated to small bulk and reprecipitated with basic lead acetate. The second lead precipitate, which was small in bulk, was united with the first lead precipitate and worked up as lead precipitate (B). The filtrate and washings (C) are dealt with later.

(a) *Treatment of Lead Precipitate (B).*—The washed lead precipitate from the first and second lead precipitations was ground up with a slight excess of dilute sulphuric acid, filtered from lead sulphate, and the excess of sulphuric acid removed from the filtrate and washings with barium hydroxide. The lead and sulphuric acid-free solution was now boiled with an excess of pure calcium carbonate for about 12 hours. Towards the end of this period a small quantity of calcium hydroxide was added, and when permanent neutrality was obtained the solution was filtered from excess calcium carbonate. The aqueous solution was evaporated and crystallised, giving rise to the following crops of crystals :—

| | gm. |
|----------------|-------|
| Crop 1 | 158·4 |
| „ 2 | 14·89 |
| „ 3 | 1·82 |
| „ 4 | 12·24 |

The sulphated ash on these fractions was determined with the following results :—

| | Per cent. |
|--|-----------|
| Crop 1.. .. . | 31·33 |
| „ 2.. .. . | 32·99 |
| „ 2 recrystallised, 1st fraction | 31·30 |
| „ 2 recrystallised, 2nd fraction | 31·21 |
| „ 3.. .. . | 33·14 |
| „ 3 recrystallised | 31·33 |
| „ 4.. .. . | { 29·26 |
| | { 30·59 |

From these figures it appears that all of these crops consist almost entirely of calcium gluconate, which has a sulphated ash content of 31·62 per cent. This was confirmed by determining the quotient :

$R \equiv \frac{\text{Calcium gluconate by polarimeter}}{\text{Calcium gluconate by sulphated ash}}$ as described on p. 344. The results obtained are given in Table XI.

TABLE XI.

| Number of Fraction. | Weight of Fraction in Gm. | Value of R $\equiv \frac{\text{Calcium Gluconate by Polarimeter.}}{\text{Calcium Gluconate by Sulphated Ash.}}$ |
|---------------------|------------------------------|--|
| Crop 1 | 158·4 | 0·982 |
| „ 2 | 14·89 | 1·042 (recrystallised, first fraction). |
| „ 2 | 14·89 | 1·025 (recrystallised, second fraction). |
| „ 3 | 1·82 | 1·073 (once recrystallised). |
| „ 4 | 12·24 | 1·028 |

Crop 1 was further shown qualitatively to consist of calcium gluconate by the following means :—

- (a) A portion was converted into the phenylhydrazide by heating with acetic acid and phenylhydrazine. The phenylhydrazide crystallised from water in white plates melting at 198° – 199° C., this melting point being unchanged on admixture with an authentic sample of gluconic acid phenylhydrazide.
- (b) An attempt was made to esterify a portion by boiling with absolute alcohol containing 2.5 per cent. dry HCl. No ester was obtained, but a small quantity of *d*-gluconic acid lactone (see FISCHER, 1890) was isolated.

The total yield of calcium gluconate is thus, $158.4 + 14.89 + 1.82 + 12.24 = 187.35$ gm. 187.4 gm. of calcium gluconate corresponds to 62.75 gm. of carbon. This amount of carbon is therefore present as gluconic acid or as lactone in 500 c.c. of the evaporated metabolism solution (solution A measuring 1,770 c.c.), which in its turn corresponds to 12.71 litres of the original metabolism solution. The amount of carbon actually isolated as calcium gluconate thus corresponds to 1.234 gm. carbon per 250 c.c. of the original metabolism solution before evaporation. If this figure is compared with the figure for carbon in non-volatile acids given in the carbon balance sheet in Table X, *i.e.*, 0.833 gm., it is obvious that only a portion of the gluconic acid present is precipitated as a calcium salt insoluble in 80 per cent. alcohol, the remainder being presumably present as a lactone.

(b) *Treatment of Filtrate and Washings (C).*—The combined filtrate and washings (C) were freed from lead by means of H_2S and evaporated to small bulk. A considerable quantity of mannitol separated, which on recrystallisation weighed 21.26 gm. and melted at 162° C. The filtrate and mother-liquors did not give rise to any further mannitol on concentration and this was shown to be due to the presence of considerable quantities of sodium acetate. In order to remove this, the theoretical amount of N/1 H_2SO_4 , necessary to decompose all the sodium acetate present, was added. The acetic acid was then distilled off *in vacuo* and a considerable quantity of sodium sulphate crystallised out and was filtered off.

An actual estimation of the total mannitol present in solution A was carried out and is described in detail below. It was thus found that 57.4 gm. of mannitol are present in 500 c.c. of solution A. Therefore, since 21.26 gm. have already been isolated, there will now be remaining in the acetic acid-free solution about 36 gm. of mannitol. Also, a total carbon estimation on this solution showed that no appreciable amount of organic material other than mannitol could be present in the solution, since its total carbon content corresponded to 50 gm. of mannitol. Hence, only about 14 gm. of any material other than mannitol were present. For this reason it was not considered worth while to investigate this fraction further.

Estimation of Mannitol.—5 c.c. of the evaporated metabolism solution (solution A measuring 1,770 c.c.) were treated by the method described in Part X for solutions

containing both glucose and mannitol. The total amount of mannitol found was 0.574 gm., corresponding to 57.4 gm. in 500 c.c. of the solution A. A companion analysis carried out by the estimation of the acetyl value of solution A gave a figure of 53.6 gm. of mannitol per 500 c.c. of solution A. Accepting as correct the figure 57.4 gm. of mannitol in 500 c.c. of the evaporated metabolism solution A, this is equivalent to 22.71 gm. of carbon or to 0.447 gm. of carbon in 250 c.c. of the original metabolism solution.

Estimation of Gluconic Acid.—An estimation was carried out of the gluconic acid present in the evaporated metabolism solution (solution A measuring 1,770 c.c.) by polarising this solution after complete neutralisation with sodium hydroxide, and calculating the gluconic acid present from an experimentally determined rotation for sodium gluconate in water.

0.2510 gm. of pure gluconic acid lactone was neutralised completely with N/1 NaOH (found, 1.43 c.c. N/1; theory=1.41 c.c.) and made up to 25 c.c. with water. This was then polarised in a 20 cm. tube using the mercury yellow light. The mean of 20 closely agreeing readings taken at intervals over 24 hours, gave $+0.335^\circ$, corresponding to $[\alpha]_{\text{Hg, yellow}} = +16.68^\circ$ for sodium gluconate weighed as lactone.

5.05 c.c. of the evaporated metabolism solution were neutralised with N/1 NaOH (0.78 c.c. needed) and made up to 50.06 c.c., filtered and polarised in a 10 cm. tube with the mercury yellow light. The mean of 10 closely agreeing readings was $+0.874^\circ$. An estimation of the glucose present, by the Wood-Ost method, indicated the presence of 0.379 per cent. glucose corresponding to a rotation of $+0.199^\circ$. Therefore, the net rotation due to sodium gluconate = $+0.675^\circ$, which corresponds to 4.406 per cent. of gluconic acid lactone, and is equivalent to 1.597 gm. of carbon present as gluconic acid or the lactone in 250 c.c. of the original unevaporated medium. This is equivalent to 64.6 per cent. of the total carbon in solution, while the "carbon as non-volatile acids" figure from the balance sheet, *i.e.*, 0.833 gm., is only 33.6 per cent. of the total carbon in solution. On the other hand, almost complete precipitation of the gluconic acid is effected by two precipitations with basic lead acetate, as is shown by the following figures:—When a portion of the original evaporated metabolism solution (A) was diluted and precipitated twice with basic lead acetate 50.0 per cent. of the total carbon in solution was precipitated in the first precipitation and 13.0 per cent. in the second precipitation, making a total of 63.0 per cent., which agrees quite well with the figure of 64.6 per cent. given above for the total gluconic acid content.

The carbon present in the original medium, either as mannitol (0.447 gm.) or as gluconic acid or gluconic acid lactone (1.597 gm.) is, therefore, 2.044 gm. of carbon per 250 c.c. medium.

From the balance sheet 0.833 gm. of carbon is present in the original metabolism solution as "non-volatile acids" and 1.411 gm. of carbon as "carbon unaccounted for," making a total of 2.244 gm. Hence, it is obvious that, at the most, only very small amounts of any products other than mannitol and gluconic acid are formed from glucose by *P. chrysogenum*. These products are present to the extent of approximately

9 per cent. of mannitol, 16 per cent. of gluconic acid as the free acid, and 16 per cent. of gluconic acid as the lactone.

Summary.

An investigation has been carried out of the metabolism products of five different species of fungi belonging to five different genera. These species were all grown on the usual CZAPEK-DOX 5 per cent. glucose solution, and had been chosen for investigation because of some interesting point in their carbon balance sheets.

(a) *Helminthosporium geniculatum*.—The main metabolic products of this species are ethyl alcohol and mannitol. Smaller amounts of glycerol and acetaldehyde were also found.

(b) *Clasterosporium species*.—The main metabolic products of this species are mannitol and ethyl alcohol. Smaller amounts of glycerol, acetaldehyde, succinic acid and probably malic acid were also found.

(c) *Aspergillus Wentii*.—The main metabolic product of this species is gluconic acid, present in solution partly as the free acid, and partly as the lactone. Smaller amounts of mannitol and glycerol were found, but no alcohol.

(d) *Fumago vagans*.—The main metabolic product of this species is gluconic acid, together with smaller amounts of glycerol and succinic acid, but no mannitol. An interesting feature of this species is the production by it, in considerable quantities, of a new polysaccharide composed entirely of glucose units. The preparation and properties of this polysaccharide are described.

(e) *Penicillium species* Ad. 11 (*P. chrysogenum*).—The main metabolic products of this species are gluconic acid and mannitol.

Studies in the Biochemistry of Micro-organisms.

PART XVIII.—*Biochemical Characteristics of Species of Penicillium responsible for the rot of Citrus fruits.*

By JOHN HOWARD BIRKINSHAW, JOHN HENRY VICTOR CHARLES and HAROLD RAISTRICK.

Decaying oranges or other citrus fruits are almost invariably infected by one of two moulds, viz., *P. digitatum* SACCARDO (= *P. olivaceum* WEHMER), which causes the olive-coloured rot, and *P. italicum* WEHMER, which is responsible for the blue-green rot. It has been found in the course of work that each of these species has very definite biochemical characteristics which are described in this paper.

1. *P. digitatum* SACCARDO (*P. olivaceum* WEHMER).

This species was first adequately described by WEHMER in "Beiträge zur Kenntnis einheimischer Pilze," Heft 2: "Untersuchungen über die Fäulnis der Früchte," Jena, 1895. Later, THOM (1910), in "Cultural Studies of Species of *Penicillium*," p. 31, renamed this species on the grounds of priority, *P. digitatum* SACCARDO.

Physiologically it is distinguished from almost all other species of *Penicillium* by the fact that while it grows readily on organic media, it either refuses to grow or grows with difficulty on synthetic media containing nitrogen as sodium nitrate (THOM, p. 32).

In the course of work described in Part IV of this series on the carbon balance sheets of species of *Penicillium*, it was found that three strains of *P. digitatum* give unusually large amounts of carbon as "Carbon in H_2SO_4 ." The strains used were the following:—

- (a) *P. digitatum*, Catalogue No. Ad. 52. This strain was purchased in 1925 from the Centraalbureau voor Schimmelcultures at Baarn under the name *P. olivaceum* WEHMER. It was sent for confirmation to Dr. THOM, who wrote, "Your No. 52, marked *P. olivaceum*, is correctly named as the olive-coloured rot of oranges, but the name should be changed to *P. digitatum* to comply with the rules."
- (b) *P. digitatum*, Catalogue No. Ad. 81. This strain was isolated at Ardeer in 1926 from a mouldy orange. It gives a very poor growth on synthetic media containing sodium nitrate, and was identified by Dr. THOM.
- (c) *P. digitatum*, Catalogue No. Ad. 102. This strain was purchased in 1927 and is the American Type Culture Collection No. 1113, isolated originally by Dr. THOM from a mouldy orange.

The carbon balance sheets prepared for these strains, which are given in Part IV,

Table III, p. 60, are all of the same type and are chiefly remarkable for the fact that each of the three strains tried gives rise to very considerable amounts of carbon in the sulphuric acid bubbler, indicating the production in fair amount by this species of some relatively volatile material. In addition, considerable amounts of "carbon in volatile neutral compounds" are produced, varying from about 20 per cent. with Ad. 52 to 30 per cent. with Ad. 102.

In order to investigate the nature of these products the following experiments were carried out: A quantity of the usual CZAPEK-DOX 5 per cent. glucose medium was made up and 350 c.c. of this placed in each of 60 1-litre conical flasks. These were plugged with cotton wool and sterilized by steaming on each of three consecutive days. After sterilization, 20 flasks were heavily sown with spores of Ad. 52, 20 were sown with Ad. 81, and 20 were sown with Ad. 102. Each flask was then fitted with a sterile rubber bung which carried two sterile glass tubes plugged with sterile cotton wool. One of these tubes reached almost to the surface of the liquid and was connected to a supply of sterile air. The other tube was cut short at the bung, and was connected to a bubbling tube which dipped beneath the surface of an absorbent solution contained in a boiling tube. In some cases this absorbent solution consisted of water, in others of normal sodium hydroxide, and in others of an aqueous solution of 2:4-dinitrophenylhydrazine hydrochloride. The whole of the flasks and fittings were incubated in the constant temperature room at 24° – 26° C. No growth was apparent in any flasks for at least a fortnight, but from that time onwards a slow growth started, and by the time the experiment terminated quite a fair growth had been obtained. Two flasks out of the 60 became infected and were replaced. Strains Ad. 52 and Ad. 102 grew rather better than strain Ad. 81.

All the flasks were sown on 25th February, 1929, and aeration was not commenced until 23rd March, 1929, from which time until the end of the incubation period about 250 c.c. of sterile air were passed through each flask each day for six days a week. At the end of the incubation period the contents of the flasks were filtered from the mycelium, each strain being dealt with separately. An average sample was taken for analysis, and a summary of the analytical results is given in Table I. The treatment of the bubblers, of the filtered metabolism solution A and of the mycelium B, are given later.

TABLE I.

| | Strain Ad. 52. | Strain Ad. 81. | Strain Ad. 102. |
|-----------------------------------|-------------------|-------------------|--------------------|
| Incubation period in days | 79 | 93 | 76 |
| | per cent. | per cent. | per cent. |
| Glucose by polarimeter | 0.650 | 1.186 | 0.985 |
| Glucose by WOOD-OST method | 0.645 | 1.098 | 0.972 |

Treatment of bubblers.—The absorbent bubblers mentioned on p. 356 were treated as follows :—

- (a) The contents of the bubblers containing water were united with solution A and worked up with this solution.
- (b) The contents of the bubblers containing normal sodium hydroxide were tested for volatile acids, but practically none were found.
- (c) The contents of the bubblers containing 2:4-dinitrophenylhydrazine remained clear throughout the experiment and indicated the absence of aldehydes or ketones.

Treatment of Solution A.—The filtered metabolism solution from each of the three strains had a very pleasant ethereal smell. The treatment of the solution was the same with each of the three strains. The solution, to which was added the aqueous absorbent solution described above, was distilled at ordinary pressure from a large bolt-head flask fitted with a long YOUNG'S fractionating column. Two fractions were collected, fraction 1 boiling at 70° C. to 98° C. and fraction 2 at 98° C. to 99° C. These fractions were now further fractionated, again using a YOUNG'S column. The following three fractions were obtained in each case :—

- (a) Boiling point, 70° to 73° C. (mainly 71° C.).
- (b) Boiling point, 78° to 81° C. (mainly 79·5° C.).
- (c) Boiling point, 88° to 98° C. (gradual rise).

The behaviour on distillation of the metabolism solution from each of the three strains was exactly similar, the weights of distillate obtained being somewhat different as is shown in Table II.

TABLE II.

| | Strain Ad. 52. | Strain Ad. 81. | Strain Ad. 102. |
|--|-------------------|-------------------|--------------------|
| Weight in gm. of fraction (a), B. Pt. 70°–73° C. ... | 6·03 | 3·07 | 4·40 |
| Weight in gm. of fraction (b), B. Pt. 78°–81° C. ... | 22·66 | 8·08 | 12·93 |
| Weight in gm. of fraction (c), B. Pt. 88°–98° C. ... | 17·23 | 4·20 | 33·80 |

Treatment of fraction (a).—Fraction (a) was shown to consist principally of *ethyl acetate* by the following means :—The whole of fraction (a) from strain Ad. 52 was shaken with about its own volume of calcium chloride solution (50 gm. CaCl₂ and 50 gm. water), separated and dried overnight over solid calcium chloride. It was distilled and gave two fractions, one weighing 1·25 gm. and boiling at 72°–74° C., while the other weighed 2·87 gm. and boiled at 75° C. (boiling point of ethyl acetate = 77° C.).

0.964 gm. of the fraction boiling at 75° C. was hydrolysed by boiling with an excess of N/1 NaOH for one hour under reflux. The excess of sodium hydroxide was titrated with N/1 nitric acid to aqueous phenolphthalein. 9.25 c.c. of N/1 sodium hydroxide were used, corresponding to an equivalent of 104 (theoretical for ethyl acetate = 88). The neutralised solution was now distilled. The distillate gave a positive iodoform reaction at 60° C. (not in the cold) and a carbon content on wet combustion corresponding to 0.517 gm. of ethyl alcohol (if the fraction boiling at 75° C. were pure ethyl acetate this amount would be 0.504 gm.). The distillation residue was evaporated to small bulk, filtered, silver nitrate added and the precipitated silver salt recrystallised from boiling water. It proved to be silver acetate. 0.2120 gm. gave 0.1363 gm. of silver on ignition, corresponding to 64.3 per cent. silver (theoretical for CH_3COOAg = 64.8 per cent.).

Hence the fraction boiling at 75° C. consists of ethyl acetate containing small amounts of ethyl alcohol, which is known to be difficult to remove entirely from ethyl acetate. The corresponding fraction (a) from strains Ad. 81 and Ad. 102 gave exactly similar results.

Treatment of fraction (b).—Fraction (b) was shown to consist principally of ethyl alcohol by the following means:—A portion of fraction (b) from strain Ad. 52 was treated with *p*-nitrobenzoyl chloride and the resultant *p*-nitrobenzoate recrystallised. It crystallised in plates and melted at 55.5°–56.5° C., and its melting point was unchanged on admixture with a sample of ethyl *p*-nitrobenzoate.

The corresponding fractions (b) from strains Ad. 81 and Ad. 102 gave exactly similar results.

Treatment of fraction (c).—Fraction (c) from each of the three strains was shown to consist principally of ethyl alcohol which was identified as the *p*-nitrobenzoate, while the higher boiling fraction consisted of water.

Treatment of Mycelium B.—The mycelium from each of the three strains, after being drained from the metabolism solution, was thoroughly extracted with boiling water, the extract from each strain being kept separate. In each case there separated from the cooled mycelium extract a white, flocculent, non-crystalline solid. A second extraction of the mycelium with boiling water gave a second crop of the same material, but the amount obtained by a third extraction was negligible. The yields of material obtained were as follows:—

| | Gm. | | | | | | |
|--------------------------------------|-----|----|----|----|----|----|------|
| Strain Ad. 52—Crops 1 and 2 together | .. | .. | .. | .. | .. | .. | 4.81 |
| Strain Ad. 81—Crop 1 | .. | .. | .. | .. | .. | .. | 2.14 |
| Strain Ad. 81—Crop 2 | .. | .. | .. | .. | .. | .. | 2.37 |
| Strain Ad. 102—Crop 1 | .. | .. | .. | .. | .. | .. | 1.97 |
| Strain Ad. 102—Crop 2 | .. | .. | .. | .. | .. | .. | 2.00 |

Properties of product from mycelium extract.

The white, flocculent, non-crystalline solid separating from the hot aqueous mycelium extract was shown to be the same material from each of the three strains and to consist in each case of a complex carbohydrate.

This carbohydrate is quite white in colour if quickly filtered and quickly dried with alcohol and ether. It gives no colour with iodine and is almost insoluble in cold water. It dissolves moderately well in boiling water, from which it separates almost completely on cooling as a white flocculent solid. The material prepared from strain Ad. 81 had the following properties :—

(1) *Optical rotation*.—The material is strongly dextro-rotatory. Because of its insolubility in cold water the optical rotation was determined in a jacketed tube at 90° C. 0.495 gm. dissolved in 200 c.c. of hot water gave $[\alpha]_{\text{Hg. green}}^{90} = +299^\circ$ and $[\alpha]_{\text{Hg. yellow}}^{90} = +270^\circ$. The material from strain Ad. 52 gave corresponding values of $+292^\circ$ and $+261^\circ$, while that from strain Ad. 102 gave $+292^\circ$ and $+266^\circ$.

(2) *Hydrolysis by boiling dilute acid*.—0.100 gm. of the material from strain Ad. 81 was heated under reflux with 10 c.c. of N/1 H₂SO₄ for three and a half hours, cooled, 10 c.c. of N/1 NaOH added and made up to 25 c.c. with water. The optical rotation of this solution calculating as glucose corresponded to a concentration of 0.400 per cent. (\equiv 0.100 gm. glucose), while glucose estimated by the WOOD-OST method gave 0.392 per cent. (\equiv 0.098 gm. glucose).

(3) *Products of hydrolysis*.—0.500 gm. of the material from strain Ad. 81 was hydrolysed for three hours under reflux with 10 c.c. of N/1 H₂SO₄ and 10 c.c. of water. At the end of the hydrolysis period the mixture was titrated to phenolphthalein with N/1 NaOH, of which 10.02 c.c. were required. Hence, it is obvious that no acidic bodies are formed by hydrolysis.

The neutralised hydrolysis mixture was made up to 50 c.c. and its glucose content estimated (a) by polarimeter = 1.009 per cent., (b) by WOOD-OST = 1.006 per cent., corresponding to yields of 0.505 gm. and 0.503 gm. of glucose from 0.500 gm. of carbohydrate hydrolysed.

It was definitely proved that glucose is the only carbohydrate formed on hydrolysis by preparing the phenylosazone from the hydrolysis mixture. The whole phenylosazone was filtered off and dried. Its melting point was 203° C. 0.1 gm. dissolved in 2 c.c. of pyridine plus 3 c.c. of ethyl alcohol gave a rotation in a 2 cm. tube of -0.29° , corresponding to -1.45° for a 10 cm. tube (NEUBERG gives -1.50° for pure glucosazone).

(4) *Effect of Enzymes*.—0.2 gm. samples of carbohydrate from each of the three strains were incubated at 25° C. with 0.2 gm. of Pangestin (commercial diastase) in 20 c.c. of water in the presence of toluene. There was no hydrolysis even after one week's incubation.

(5) *Analysis by combustion*.—A sample of the carbohydrate from Ad. 81, dried to constant weight *in vacuo* at 50° C. over P₂O₅, was analysed by SCHOELLER, Berlin, with the following results :—

TABLE III.

| Weight of Substance analysed. | Weight of CO ₂ . | Weight of H ₂ O. | Percentage Carbon. | Percentage Hydrogen. |
|--|-----------------------------|-----------------------------|--------------------|----------------------|
| mgm. | mgm. | mgm. | per cent. | per cent. |
| 4·855 | 7·800 | 2·83 | 43·82 | 6·52 |
| 4·481 | 7·165 | 2·58 | 43·76 | 6·47 |
| Theoretical for C ₆ H ₁₀ O ₅ | — | — | 44·42 | 6·22 |

2. *P. italicum* WEHMER.

This species was first described by WEHMER (*loc. cit.* p. 68), and is further described by THOM (*loc. cit.* p. 29). WEHMER showed that the blue-green rot of citrus fruits is a different species from the similarly coloured apple rot. THOM (*loc. cit.* p. 31) says "Pure cultures of *P. italicum* WEHMER can always be secured by finding decaying oranges in the market which have the blue-green areas of rot just beginning to appear upon them." During the course of other work it was observed that a reputed culture of *P. italicum* gave a characteristic colour reaction with ferric chloride. This appeared of sufficient interest to warrant the extension of the test to other cultures of *P. italicum* obtained from different sources and it has been found that the tests to be described are given by all genuine cultures of *P. italicum* which were used.

The tests, which are described later, were given by all the following strains of *P. italicum* WEHMER :—

- (a) Catalogue No. Ad. 17. Isolated at Ardeer in 1925 from a mouldy lemon.
- (b) Catalogue No. Ad. 84. Isolated at Ardeer in 1926 from a mouldy orange.
- (c) Catalogue No. Ad. 85. Isolated at Ardeer in 1926 from a mouldy orange.
- (d) Catalogue No. Ad. 86. Isolated at Ardeer in 1926 from a mouldy orange.
Cultures Ad. 84, Ad. 85 and Ad. 86 have all been identified by Dr. THOM as strains of *P. italicum*.
- (e) Fourteen other strains of *P. italicum* isolated from mouldy oranges and lemons obtained from markets in different parts of Great Britain, in order to ensure differences in strains.

Single spore cultures of each of the above 18 strains of *P. italicum* were prepared and used for inoculating tubes, each containing 10 c.c. of the modified CZAPEK-Dox glucose medium containing twenty times the amount of ferrous sulphate given in Part I, p. 7.

The medium was sterilized by steaming for half an hour on each of three consecutive days. After inoculation the tubes were incubated in the dark at 24° C. At intervals of 6 days, 15 days, 20 days and 40 days, one tube of each culture was tested, and while it was found that not all cultures gave a positive result in 6 days or 15 days, each of the 18 strains tried gave a definite positive result after 40 days' incubation.

The test was carried out as follows :—The metabolism solution, without sterilizing, was poured from the mycelium and filtered through a small filter paper. The filtrate was then divided into two equal parts which were treated as follows :—

(a) To one-half was added a single drop of 10 per cent. ferric chloride solution in water. A transient green colour is produced when the ferric chloride comes in contact with the solution, but this colour disappears on shaking. Further addition of ferric chloride solution drop by drop, until a slight excess has been added, gives rise to a permanent, beautiful, emerald-green colour which varies in intensity with different strains, some of the strains giving a very intense reaction. If this green solution is now diluted to such an extent that the green colour is only just obvious, and two or three drops of a freshly prepared aqueous solution of potassium ferricyanide are now added, a deep blue colour is immediately obtained indicating the reduction of the ferric chloride to the ferrous state. In our opinion this is due to the oxidation by the ferric chloride of some metabolic product of *P. italicum*, with the formation of (a) ferrous chloride which now reacts with potassium ferricyanide to give the Prussian blue colour, and (b) an oxidation product which now gives a deep emerald-green colour with ferric chloride.

(b) To the other half of the filtered metabolism solution was now added, drop by drop, a filtered saturated solution of bleaching powder, with shaking between the additions. In every case a copious white precipitate was formed and a purple colour was produced, varying in intensity from a pale amethyst to a permanganate colour. With subsequent addition of excess of bleaching powder solution this purple colour disappears, leaving a colourless solution. It was noticed that the intensity of the ferric chloride reaction runs parallel with the intensity of the purple colour given with bleaching powder, and it is proposed to attempt the isolation and chemical investigation of the product which is responsible for the above colour reactions.

We are of the opinion that these colour reactions are not only characteristic of, but specific for *P. italicum* WEHMER, since out of a large collection of different species of different genera, *P. italicum* WEHMER is the only mould in our possession which gives the colour reactions above described. The reaction given by *P. italicum* with ferric chloride might be confused with the somewhat similar reaction given by *Citromyces* species and described in Part XI. There are two very definite differences. In the first place the *Citromyces* colour is developed *immediately* with ferric chloride, while the colour with *P. italicum* requires *an excess* of ferric chloride. Further, *Citromyces* species do not give the characteristic purple colour with bleaching powder, which as described above is given by *P. italicum*.

Summary.

The biochemical characteristics are described of two species of *Penicillium* responsible for the rot of citrus fruits, viz., *P. digitatum* SACCARDO (*P. olivaceum* WEHMER) and *P. italicum* WEHMER.

P. digitatum SACCARDO is unique amongst species in our collection in the fact that it produces from glucose considerable amounts of ethyl acetate. In addition it also produces ethyl alcohol and a new polysaccharide which gives rise to glucose on hydrolysis.

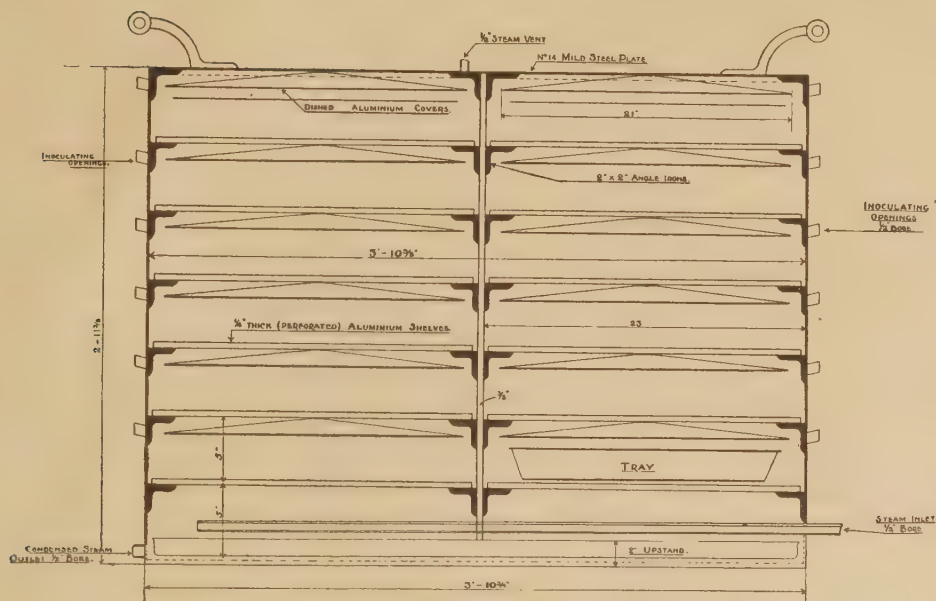
P. italicum WEHMER produces from glucose a new metabolic product which is characterized by its colour reactions with ferric chloride and bleaching powder. These colour reactions are characteristic of and diagnostic for *P. italicum* WEHMER.

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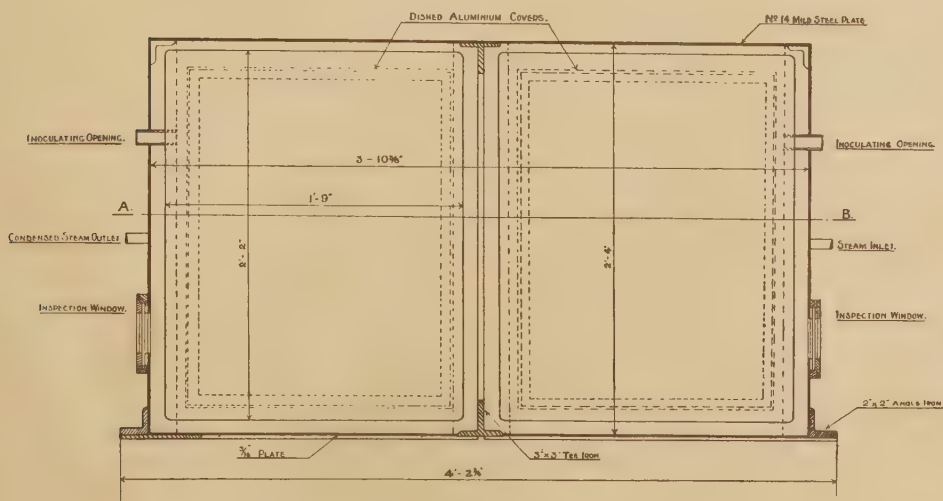
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STERILIZER-INCUBATOR (p. 136).
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STERILIZER-INCUBATOR (p. 136).
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